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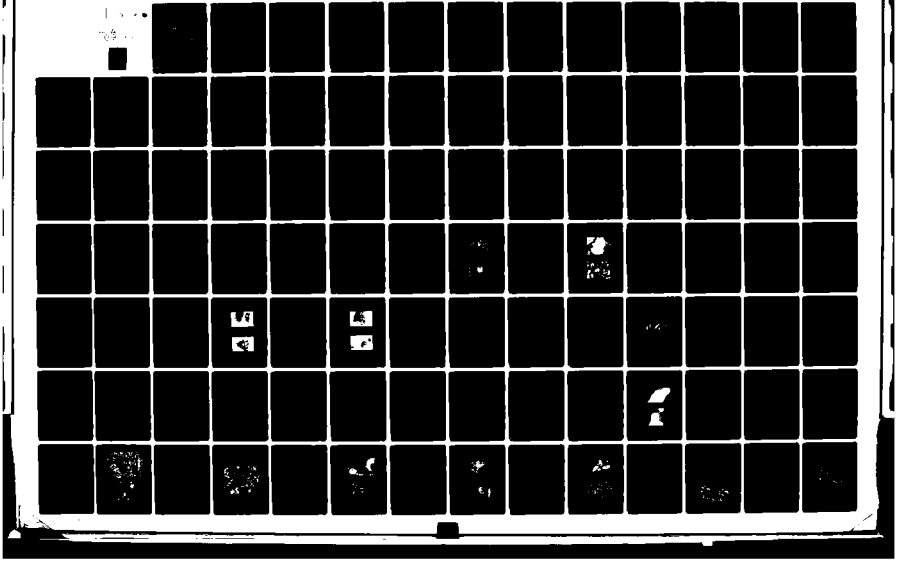

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ABSTRACT OF DISSERTATION

THE MORPHOGENESIS AND BIOLOGY OF

A MORBILLIVIRUS FROM MCF CATTLE

Submitted by: Gary R. Coulter, Major, USAF, for the degree of Doctor of Philosophy; Summer, 1979. Colorado State University, 162 pp.

Cell-associated syncytiogenic viruses were previously isolated from a calf (72-P-535) with experimentally induced malignant catarrhal fever. The isolate recovered from leukocytes was investigated by studying its growth and cytopathology in low passage fetal bovine cells. Antigenic studies with this virus and other 72-P-535 isolates using direct and indirect immunofluorescence techniques revealed that each isolate was related to agents of the measles, canine-distemper, rinderpest group.

The 72-P-535 leukocyte isolate induced polykaryons in several fetal bovine cell types but not in HeLa, VERO or mouse L-cells. Neither nuclear nor cytoplasmic inclusions were detected in Giemsa-stained coverslip preparations of infected cells.

Guinea pig, human 'O' and bovine erythrocytes were not agglutinated by cell-free culture fluids or cell lysates nor did they absorb to infected cells.

Pleomorphic enveloped virus particles 150-500 nm in diameter were detected in negatively stained preparations of cell-free culture fluids. These particles contained striated

nucleocapsids 17-20 nm in diameter. Similar membrane-associated nucleocapsids with a buoyant density of 1.25-12.7 gm/cm^3 were the only virus specific structures detected in negatively stained preparations of disrupted infected cells.

The morphogenesis and cytopathology of the leukocyte isolate was investigated using bovine fetal spleen monolayers which were fixed and embedded in situ to preserve the spatial interrelationships of virus and host cell components. Nucleocapsids associated with a granulofibrillar material (fuzzy nucleocapsids) were first detected in the perinuclear region and later throughout the cytoplasm of infected cells. Nucleocapsids devoid of associated material (smooth nucleocapsids) were detected both in the cytoplasm and nuclei of degenerating cells. Enveloped extracellular particles formed by the evagination of modified host cell plasmalemma were 150-500 nm in diameter, highly pleomorphic and contained nucleocapsids corresponding to those detected in negatively stained preparations. Defective or incomplete particles were also detected which were empty or contained host cell components. An external fringe of 9-11 nm was observed on some particles but was frequently discontinuous or absent.

Experimental MCF was serially transmitted through 12 passages in cattle. Cell-associated virus isolation techniques were applied to tissue and fluid samples obtained at necropsy. The results of the isolation procedures and preliminary immunofluorescence and electron microscopic identification of some of the isolates are reported.

DISSERTATION

THE MORPHOGENESIS AND BIOLOGY OF A MORBILLIVIRUS FROM MCF CATTLE

Submitted by

Gary R. Coulter

Department of Microbiology

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer, 1979

COLORADO STATE UNIVERSITY

SUMMER, 1979

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY GARY R. COULTER ENTITLED THE MORPHOGENESIS AND BIOLOGY OF A MORBILLIVIRUS FROM MCF CATTLE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY .

Committee on Graduate Work

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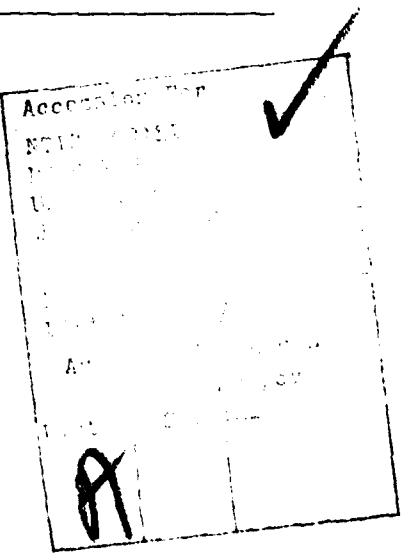

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canine-distemper virus antiserum; Dr. Max J. Appel of the James A. Baker Institute for Animal Health, Cornell University, (3) rinderpest virus antiserum; Drs. J. J. Callis and A. H. Dardiri of the Plum Island Animal Disease Center (USDA), (4) bovine syncytial virus antiserum; Dr. W. A. Malmquist of the National Animal Disease Laboratory (USDA), and (5) bovine respiratory syncytial virus antiserum; Dr. M. H. Smith of Montana State University.

A major part of any graduate program is the interaction one has with fellow students. In this regard, I consider myself most fortunate. I can only begin to express my sincere appreciation to Drs. Jeff and Kathryn Leary, Mike Parker, Ibrahim Hajer, Priscilla Spears and so many others for the comeradery and friendship they have given me.

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Finally, a special thank you to my secretary, Mrs. Betty Plonsky, and my typist, Ms. Sue Waterbury, for the endless hours of typing, revision and retyping of which this dissertation is the culmination.

DEDICATION

For the encouragement, support and understanding which has sustained me through these past four years, this study is gratefully dedicated to my wife, Pat, my son, Chris and my daughter, Katie.

TABLE OF CONTENTS

INTRODUCTION

The acute, infectious and highly fatal clinicopathological syndrome known as "malignant catarrhal fever" (MCF) affects cattle (Pierson, 1973a; Murray and Blood, 1961; Gray and Arthony, 1968), deer (Huck et al., 1961; Clark et al., 1970; Sanford et al., 1977), bison (Ruth et al., 1977) and other ruminants throughout the world Plowright, 1964). The form observed in East Africa is more properly termed a disease rather than a syndrome since the etiological agent has been identified as bovid herpesvirus 3 (Plowright et al., 1960, 1965a; Wibberley, 1976). The African MCF virus which is naturally transmitted to cattle from inapparently infected wildebeest, is syncytiogenic and was cell-associated upon initial isolation (Plowright, et al., 1960). Both cell-associated and cell-released virus caused MCF after inoculation into cattle. Although clinically and pathologically indistinguishable from the African form of MCF, the etiological agent has not yet been identified for the European or North American forms. Regardless of locale, MCF is considered to occur sporadically within a herd although several epizootics were reported (Pierson et al., 1973b; James et al., 1975; Orsborn et al., 1977). During the winter of 1971-1972, a severe epizootic of MCF occurred in a herd of Colorado feedlot cattle wherein 87 of 231 cattle (37%) died within 68 days (Pierson et al., 1973a).

During this epizootic, Storz and coworkers obtained blood and tissue samples from cattle affected with experimental or naturally-occurring MCF. Twenty-two strictly cell-associated syncytiogenic viral strains were isolated by co-cultivating trypsin-dispersed cells from infected tissue preparations with low passage fetal bovine spleen (BFS), thyroid (BFThyr) or adrenal (BFAdr) cells (Storz et al., 1976). Virus was isolated from leukocytes, ependyma, lymph node, spleen, kidney, adrenal and thyroid glands. Although isolates from some animals were not maintained for extended periods, the isolates recovered from experimentally infected calf designated 72-P-535 were preserved. The virus isolated from leukocytes was strictly cell-associated, and produced polykaryons in a variety of bovine and ovine cell cultures. Neither nuclear nor cytoplasmic inclusions were observed in Giemsa-stained infected BFS monolayers. Cattle inoculated with approximately 10^7 BFS cells from 72-P-535 infected monolayers developed a transient pyrexia but no other clinical signs. The experimental cattle were not tested for previous exposure to this type of infectious agent because reliable tests were not available.

Based on cytologic findings and electron microscopic analysis of infected culture fluids, Okuna (1974) tentatively identified the 72-P-535 leukocyte isolate as a bovine syncytial virus (BSV) (Malmquist et al., 1969) but he could not demonstrate an antigenic relationship. Although previously isolated from cattle with MCF (Clarke et al., 1973), BSV has

not been directly associated with disease. Its presence was reported to interfere with the isolation or mask the presence of other cell-associated syncytiogenic viruses present (Van der Maaten et al., 1972).

Storz and coworkers (1976) observed that multiple mixed infections involving cell-associated viruses should be expected. Problems inherent in the separation and identification of viruses under such conditions are compounded when infectivity is strictly cell-associated. With this in mind, work was begun on the exact identification and characterization of the 72-P-535 isolates.

The objectives of this study were:

1. To identify and compare each of the seven isolates obtained from experimental calf 72-P-535.
2. To determine whether the seven isolates recovered from calf 72-P-535 represent the same virus.
3. To test for multiple mixed infection in these isolates.
4. To describe the cytology and cytopathology of polykaryons induced in low passage bovine fetal cells in culture.
5. To determine the host cell range of a representative isolate and to compare its kinetics of growth and polykaryon formation in several cell types.
6. To define ultrastructurally the morphogenesis of a representative isolate and to relate virus morphogenesis with observable alterations in the structure and function of cellular organelles.

7. To correlate virus morphogenesis with sequential steps of polykaryon formation and to describe the mechanism of polykaryocytosis.

Experiments performed to reach the stated objectives and the results of these investigations are reported in the following major chapters. Part II has been submitted for publication to the American Journal of Veterinary Research and Part III is being revised prior to submission to Archives of Virology.

Part I

Review of Literature Concerning

Malignant Catarrhal Fever: The Disease and its Etiology

Introduction

Malignant catarrhal fever (MCF) is an acute infectious disease of cattle and other ruminants throughout the world (Plowright, 1968). Outbreaks have been reported in Japan (Fujimoto et al., 1958; Ohshima et al., 1977), the Australian subcontinent (James et al., 1975; Pearson, 1956), Africa (Piercy, 1954), Europe (Selman et al., 1974) and North America (Pierson et al., 1973a; Orsborn et al., 1977). In North American, MCF occurs as a sporadic or epizootic disease in range (Gray and Anthony, 1968), feedlot (Pierson et al., 1973a) and dairy cattle (Murray and Blood, 1961; Orsborn et al., 1977). Deer (Pierson et al., 1974; Huck et al., 1961; Clark et al., 1970) and the American bison (Ruth et al., 1977) are also affected.

The morbidity within a herd is usually low, the disease affecting one or a few animals (Roderick, 1958). Case fatality rates may exceed 95% (Plowright, 1968). Cattle of all ages, sexes and breeds are at risk (Piercy, 1952; Jensen and Mackey, 1972). Although known to the Masai tribesmen of East Africa since before recorded history, MCF was first reported from Africa and Europe late in the 18th century (Colson, 1930). Around 1913, it was first reported in North America. Regardless of locale, the symptomatology, gross and histopathological characteristics are similar and serve as the basis for diagnosis (Daubney and Hudson, 1936; Plowright, 1968; Jubb and Kennedy, 1970). Liggitt (1978a) and colleagues (1978b, 1979a, 1979b, 1979c) recently

summarized the pathology and pathogenesis of the North American form in experimental cattle and should be consulted for details. Gotze (1930) first described the four clinically recognizable forms of the disease: (1) peracute, (2) head and eye, (3) intestinal and (4) the mild or benign form. While this classification is based on the primary nature of the clinical involvement, certain key characteristics are common to all forms. In North America, the peracute form is characterized by hemorrhagic gastroenteritis, diarrhea, high fever and early mortality (Ohshima et al., 1977). The head and eye form is the classical form of MCF and is seen both in natural and experimental infections (Kalunda, 1975). This form is frequently observed in cattle when the disease runs a prolonged course. Clinical signs include bilateral panopthalmitis, keratitis and ocular opacity, severe mucous membrane and occasional central nervous system involvement. The intestinal form is generally subacute. Head and eye signs are minimal although fever, dairrhea and lymphadenopathy are common. The mild form is frequently self limiting and often observed in experimental animals. Signs which are characteristic of the intestinal and head and eye forms frequently occur together.

Regardless of the primary clinical involvement, the signs which, taken together, are most indicative of MCF include: high fever, diarrhea, catarrhal rhinitis, corneal opacity, lacrimation and photophobia, ulcerations and sloughing of the oral and nasal nucosae, conjunctival and

scleral hyperemia, lymphadenopathy and sudden death (Piercy, 1952; Plowright, 1968; Selman et al., 1974).

Malignant catarrhal fever is a pansystemic disease and is characterized by lymphoproliferations and gross pathological changes observable throughout the gastrointestinal system as well as in lymphoid, reticuloendothelial and parenchymal organs (Liggitt, 1978a). Lesions vary from focal areas of hyperemia to frank ulcerations and erosions of the oral, nasal and esophageal mucosae, hard palate and cheek papillae. Tonsils, Peyer's patches and lymph nodes are consistently enlarged.

Characteristic histological changes are observed in vascular, epithelial and lymphoid elements including generalized lymphoid and reticuloendothelial hyperplasia and mononuclear cell infiltrations of nearly all organs, a non-suppurative meningo-encephalitis and necrosis of the epithelium and underlying tissues.

Early investigations centered on the symptomatology, pathology, transmission and epizootiology of MCF. Mettam (1923) investigated a disease in South Africa known to the Boers as 'snotsiekte' (snot sickness) and established a firm basis for its further investigation and comparison. He documented the previously unsupported observation that snotsiekte was more frequent in cattle associated with calving or neonatal wildebeest (wildebeest-associated MCF) and that the disease was not transmissible between cattle by contact. After using whole blood to successfully transmit the disease

between cattle and from inapparently-affected wildebeest to cattle, he proposed that the etiological agent was an erythrocyte-associated virus transmitted by a hematophagous insect. Based on his clinicopathological findings, Mettam concluded that snotsiekte and MCF were different disease entities, an idea later refuted by many investigators.

Götze (1930) and Liess (1929, 1930) were able to transmit MCF to 13 of 34 cattle inoculated with bacteria-free whole blood and, more importantly, implicated sheep as inapparently-affected reservoirs of MCF outside Africa (sheep-associated MCF). They agreed with Mettams's (1923) earlier hypothesis concerning the etiology of MCF, but considered snotsiekte and MCF the same disease.

The only reported direct cattle to cattle contact transmission of MCF was described by Daubney and Hudson (1936) who also transmitted MCF to 33 of 36 cattle using defibrinated blood, lymph node or brain tissue homogenates. They also transmitted MCF to cattle after first establishing the disease experimentally in rabbits. Their report of transmitting the disease to sheep and then back to cattle has not been substantiated. In 1938, duToit and Alexander summarized the available information concerning MCF and concluded that MCF and snotsiekte were probably the same disease caused by an erythrocyte-associated virus and that wildebeest were undoubtedly implicated in the transmission of the African form of the disease. The evidence concerning the role of sheep was considered contradictory and, thus, inconclusive at

that time. Piercy (1952) demonstrated that the virus responsible for the wildebeest-associated form was associated with the leukocyte rather than the erythrocyte fraction of blood and that the disease was not directly transmissible to cattle by contact. Inoculations with as little as 0.05 ml of leukocytes were sufficient to pass the disease in cattle whereas filtered blood or erythrocytes were not infectious.

Indirect evidence of viral causation was supplied by researchers who detected inclusions in tissues of diseased animals. Goss and colleagues (1948) demonstrated basophilic and acidophilic cytoplasmic inclusions in epithelial and other cells from MCF animals. Stenius (1952, 1953) reported intracytoplasmic inclusions in various portions of a brain which he concluded were "probably due to intracellular virus reproduction." Danskin (1955) observed inclusions within cells of the spleen, lymph nodes and lungs of an affected animal. Roderick's (1958) observation that, "Inclusions bodies are evidently as typical for MCF as Negri bodies for rabies" was followed closely by Berkman and colleagues' report (1960) that single and multiple inclusions were scattered throughout the neuroplasm of affected animals. Since 1960, there have been no reports of cellular inclusions in tissues from MCF animals except Ohshima and colleagues' report of inclusions within parietal cell nuclei (1977). Both Plowright (1968) and Liggitt (1978a) reported that virus-specific structures were not observable within infected tissues at either the light or electron microscopic level.

Based on our current knowledge of the disease and the likli-hood of multiple infections, the possibility that the described inclusions were caused by the etiological agent of the wildebeest-associated form of MCF appear remote. Definitive proof of a viral etiology required the isolation and identification of an agent from MCF cattle, sheep or wildebeest which could induce the disease upon inoculation into cattle. Mansjoer (1954) reported isolating a virus from a case of MCF in water buffalo. The agent was cultured in chick embryos and could be propagated in rabbits. The virus was viable after remaining at room temperature for 10 days, at -20 C for 30 days or after lyophilization and storage at -20 C for one year. Since the clinical and pathological features of the disease were not described, it is questionable whether the disease affecting the water buffalo was actually MCF.

The studies of Plowright and his associates have added immeasurably to our current knowledge of the etiology, pathology, natural and experimental routes of transmission of the wildebeest-associated form of MCF. In Africa, Plowright and associates (1960) isolated a cell-associated virus (AMCFV) by direct cultivation of tissues from near term wildebeest fetuses. Leukocyte and lymph node cell suspensions also yielded virus after co-cultivation on bovine thyroid monolayers. Since Plowright had previously demonstrated the close affinity of the virus for lymphoid tissues, blood, spleen and lymph node suspensions from inapparently

infected wildebeest were inoculated into cattle with positive results. Once successfully transmitted to cattle, subinoculations using as little as 5 ml of blood or 0.5 ml of lymph node suspensions were accomplished.

Upon initial isolation, the virus was strictly cell-associated, syncytiogenic and produced Feulgen positive, Cowdry type A intranuclear inclusions in bovine thyroid monolayers (Plowright et al., 1960, 1965a). Virus replication was inhibited by iododeoxyuridine (IUdR) but not by fluorodeoxyuridine (FUdR) (Plowright et al., 1965a). Neither inclusions nor polykaryons were ever observed in vivo (Plowright, 1968) and parenteral inoculation of infected tissue culture cells produced MCF in cattle (Plowright et al., 1960). Some strains produced cell-released virus after 7-30 tissue culture passages and exhibited reduced virulence for cattle. The released virus was neutralized by covalescent bovine serum or serum from free-living populations of wildebeest, hartebeest, topi or oryx (Plowright, 1965c; Reid et al., 1975). Recently Reid and Rowe (1973) isolated a similar virus from the hartebeest.

The virus is maintained as a clinically silent infection of black wildebeest, blue wildebeest, hartebeest and, based on seroconversion data, the topi and the oryx (vide infra). Evidence for the vertical (transplacental) transmission of the virus by wildebeest and cattle has been obtained (Rweyemamu et al., 1976; Plowright et al., 1972). Neonatal wildebeest are highly viremic. Colostral antibody delays

but does not protect calves infected in utero (Plowright et al., 1972). The natural route of transmission from wildebeest to cattle has not yet been ascertained although RWeyemamu and colleagues (1974) demonstrated the infectivity of wildebeest nasal secretions and afterbirth.

Based on electron microscopic and cytologic investigations of its morphology, morphogenesis and cytopathology, the AMCFV has been classified as bovid herpesvirus type 3 (Plowright, 1965a; Wibberley, 1976). Other related viruses include Epstein-Barr virus, cytomegalovirus, varicella-zoster and Marek's disease virus. In light of the transmission, isolation and retransmission studies described, the etiological role of this virus with respect to wildebeest-associated MCF is considered unequivocable.

Based on the several similarities which exist among the African, European and North American forms of MCF, Plowright (1968) suggested that all the forms were caused by the same or similar agents. Since no etiological agent or reservoir host has unequivocably been shown to be associated with MCF outside of Africa, the basis for this suggestion requires careful examination. Contact transmission has not been documented in Europe or North America, therefore, the presence of a reservoir host has been inferred. Since Mettam's (1923) and Götze and Leiss' (1929) original observations, many reports have implicated sheep as inapparently-infected reservoirs both inside and outside of Africa (Daubney and Hudson, 1936; deKock and Neitz, 1950; Stenius, 1952, 1953; Piercy,

1954; Berkman et al., 1960; Murray and Blood, 1961; Plowright, 1964, 1965; Pierson et al., 1973a; Sanford et al. 1977; Reid et al., 1979). The association of MCF outbreaks with lambing ewes (Murray and Blood, 1961; Gray and Anthony, 1968; Pierson et al., 1973a) closely parallels the situation with calving wildebeest (Daubney and Hudson, 1936; Boever and Kurka, 1974). Furthermore, clinicopathological characteristics of the sheep and wildebeest-associated forms of MCF are very similar (Plowright, 1968; Liggitt, 1978a; and colleagues, 1978b). Although the AMCFV may have a sheep reservoir in North America and Europe, major differences with respect to its ability to establish infections in laboratory animals, its efficiency of transmission between cattle and ease of virus isolation suggest that different etiological agents may be involved.

The wildebeest-associated disease is readily transmitted to susceptible cattle using 0.5-5.0 ml of blood or lymph node homogenates (Piercy, 1952; Plowright, 1968; et al., 1960, 1963). Although several successful transmissions have been reported, the success rate is considerably lower and more sporadic using samples from cattle with the sheep-associated form (Götze, 1930; Blood et al., 1961; Pierson et al., 1974; Selman et al., 1974, 1978; Liggitt et al., 1978b). Even when successfully transmitted by blood inoculation, volumes required ranged from 500-1500 ml (Selman et al., 1978; Liggitt et al., 1978b).

Parenteral inoculation of infected blood or tissue samples from cases of wildebeest-associated MCF readily induced a disease in rabbits similar to that seen in cattle (Piercy, 1955; Plowright, et al., 1960, 1972). Kalunda (1975) confirmed these reports and extended the host range to include hamsters. In contrast, most attempts to simulate MCF in rabbits using samples from sheep-associated cases have been unsuccessful (Stenius, 1952, Blood et al., 1961; Pierson et al., 1975a). In Arizona, Mare (1977) transmitted a disease similar to MCF to rabbits using buffy coat cells from an MCF animal. It is uncertain whether the inoculum was actually from a case of sheep-associated MCF since the cattle herd originally involved was penned near a zoological park containing several species of African ungulates.

Plowright (1964) was unable to isolate a virus from cases of sheep-associated MCF using techniques proven reliable in isolating the wildebeest-associated herpesvirus. Both attempts to isolate a sheep-associated virus reported in 1961 failed (Huck et al., 1961; Blood et al., 1961). Selman and coworkers (1974), using Plowright's isolation procedures, were unsuccessful at recovering virus. Horner and colleagues (1975) co-cultivated leukocytes and kidney cells from affected cattle with bovine thyroid monolayers for up to 100 days, but were unable to recover virus. Sanford and coworkers (1977) were also unsuccessful at isolating virus from splenic tissues of affected Sika deer. Ruth and associates (1977) applied co-cultivation and direct

cultivation techniques to samples from affected bison without success. In the most recent report, Reid and coworkers (1979) used homogenates of a variety of cervid tissues cultivated with porcine kidney monolayers but were unable to isolate a virus. The direct cultivation of kidney, lymph node and adrenal cells from cervids with MCF was also unsuccessful.

Such evidence does not preclude the possibility that a herpesvirus similar to the AMCFV is responsible for MCF in Europe and North America. The biological characteristics of such a variant must, however, be sufficiently different from the parent strain as to render its isolation more difficult thereby hindering progress in establishing the etiology of MCF outside of Africa.

Although their possible etiologic roles have not yet been determined, herpesviruses have been isolated from MCF cattle both in the United States and in Iran. Ramyar and Hessami (1972) recovered a cell-released herpesvirus from the mesenteric lymph nodes and spleens of affected Iranian dairy cattle. The virus was propagated in primary calf kidney monolayers, remained viable after freeze-thawing and was neutralized by antiserum to the AMCFV. No details of cytopathic effect or animal inoculations were reported. In a preliminary report, researchers at the Plum Island Animal Disease Laboratory (PIADL) recently disclosed that they isolated a herpesvirus from blood and tissue samples obtained during a recent MCF outbreak in Minnesota (Hamdy and Dardiri,

1978a; et al., 1978b; USDA, 1978). The virus did not induce MCF in susceptible cattle although it was neutralized by antiserum to the AMCFV and was proven antigenically similar to the same using the complement fixation, immunofluorescence and immuno-electron microscopic techniques. No conclusions can be drawn with regard to its etiological role in sheep-associated MCF until further details of its cytologic and pathologic characteristics are reported.

Storz (1968) and coworkers (1976) isolated a cytolytic cell-released herpesvirus from the spleen of a classical case of bovine MCF. After five tissue culture passages in bovine fetal spleen (BFS) monolayers released virus titers of $10^{3.25}$-$10^{5.3}$ were obtained. Cytopathic effects (CPE) were not detected in bovine fetal testicle or kidney monolayers although titers of $10^{4.3}$-$10^{4.9}$ were measured in culture fluids after the cells were disrupted. The virus (66-P-347) was not syncytiogenic but produced Cowdry type A inclusions in infected cells within 15 hours after inoculation. More recently, Storz and associates (1977) isolated a herpesvirus from tissue samples from a bison affected with MCF. The virus was recovered from adrenal glands and spleen by co-cultivation with BFS cells. Direct cultivation of thyroid and spleen was also successful. The virus (75-P-2756) was cell-associated and syncytiogenic. Cytoplasmic inclusions observed electron microscopically were similar to structures reported within cells infected by herpesviruses of the cytomegalovirus group (Storz and Todd, 1979). Until these

isolates are further characterized, a herpesvirus etiology for the North American or European forms of MCF should not be assumed. Since different etiological agents may act through similar pathogenic mechanisms, the biological characteristics and disease potential of each virus isolated from MCF cattle should be evaluated independently and objectively.

In Northern Ireland, Clarke and associates (1973) isolated a virus from a field case of MCF which was syncytiogenic and later identified as a bovine syncytial virus (BSV) (Dermott et al., 1971; Van der Maaten et al., 1973). Storz and coworkers (1977) recovered a virus related to BSV from a case of MCF in an American bison. Although repeatedly isolated from cattle with lymphosarcoma, BSV has also been isolated from clinically normal cattle and has never been directly implicated in the etiology of a disease (Malmquist et al., 1969; Boothe et al., 1970; Estes et al., 1970). Scott and associates (1973) reported that BSV is ubiquitous within the cattle populations of the world.

Although Clark and colleagues (1976) reported a togavirus within cells of affected Texas cervids, their scant electron microscopic evidence does not fully substantiate their claim.

During an epizootic of MCF in Colorado, Storz and coworkers recovered both cell-associated and cell-released viruses from tissues of affected feedlot cattle (Pierson et al., 1973a; Storz et al., 1976). Both a cell-released parvovirus and an unidentified cell-associated virus were

recovered from experimental animal 72-P-293. Additional cell-associated isolates were obtained from leukocytes, ependyma, spleen lymph node, kidney and other tissues of experimental cases 72-P-462 and 72-P-535. The cell-associated isolates from 72-P-293 and 72-P-462 were not preserved. The 72-P-535 isolates were preserved and are the subject of this investigation.

The inability of many researchers to isolate cell-associated syncytiogenic viruses from MCF animals may be due to the special conditions required for their isolation. Procedures regularly employed in the isolation of cell-released viruses include inoculating established monolayers with fluids from tissue samples in which the cells have been mechanically or osmotically disrupted. Inoculated monolayers are frozen and thawed between passages and fluids free of disrupted cell components are used as inocula through successive subpassages. Infected culture fluids can usually be maintained by short term storage at -70 C.

In contrast, Storz and colleagues (1976) used very different techniques to recover the 72-P-535 and other cell-associated isolates. Cells were obtained from tissue samples by trypsinization rather than disruptive homogenization procedures. Both stationary phase and actively growing monolayers of bovine fetal spleen, thyroid and adrenal cells were inoculated with living (infected) cells. Suspensions of infected and uninfected cells were mixed and allowed to form a monolayer. After dispersing infected monolayers with

trypsin-EDTA, subpassages were made using living cells as inoculum. Freeze-thawing and other disruptive procedures were avoided. If polykaryons were detected, isolates were passaged once more and then stored under liquid nitrogen in media containing either dimethyl sulfoxide (DMSO) or glycerol with the addition of 15% lamb serum. Some Colorado isolates subsequently became cell-released and cytolytic after serial tissue culture passage. The methods employed to isolate cell-associated viruses are compared with those used to isolate cell-released viruses in Table 1. The Colorado isolates which remained cell-associated induced polykaryons in low passage bovine fetal monolayers (Storz et al., 1976). Roizman (1962) observed that polykaryocytosis is a cytopathic effect common to many groups of infectious agents including most groups of enveloped viruses. Extensive research into the mechanism of virus-induced cell fusion was begun after Okada's (1958) report that high multiplicities of ultraviolet light-inactivated Sendai virus (hemagglutinating virus of Japan), a paramyxovirus would induce the fusion of cultured mammalian cells. Bratt and Gallaher (1972) identified two basic types of virus-induced cell fusion; fusion from within (FFWI) and fusion from without (FFWO). Table 2 compares the processes. Sendai virus and the other paramyxoviruses are the classical models for FFWO while herpesviruses constitute the most frequently described example of FFWI.

Table 1. Comparison of Cell-Related and Cell-Associated Associated Virus Isolation Techniques

Cell-Free Virus	Cell-Associated Virus
Tissues homogenized and cells disrupted	Cells dispersed by trypsin digestion; not disrupted
Established monolayers inoculated	Both stationary phase and growing monolayers inoculated: Co-cultivation with uninfected cell suspensions and direct cultures of infected tissues employed
Samples frozen and thawed or otherwise disrupted between passages	Passages made with living cells: No freeze-thawing or other disruptive procedures
Monolayers and culture fluids frozen at -70 C	Infected cells frozen in media containing DMSO or glycerol and serum then stored under liquid nitrogen

Table 2. Comparison of FFWO and FFWI (After Bratt and Gallaher, 1972)

FFWO	FFWI
Virus may be infectious, non-infectious, reassembled envelope fragments or cell-free supernatant fluids	Infectious virus only
May be detected early (30 minutes - 3 hours)	Detected late (6 hours - several days)
Requires high MOI	Requires low MOI; often inhibited if high MOI used
Does not require DNA-dependent RNA synthesis (Actinomycin D does not inhibit)	Same unless virus has DNA-dependent nuclear phase
No requirement for virus-specific protein synthesis	Absolute requirement for virus-specific protein synthesis
Caused by both virulent and avirulent strains	Best manifested by virulent strains of virus

Regardless of the type of fusion induced, the chemical nature of the viral "fusion factors" (Kascardo and Karzon, 1965) appear to be chemically and functionally similar. Evidence suggests that virus-induced membrane fusion of either type depends upon the presence of a specific viral glycoprotein (Shimizu et al., 1976; Levitan and Blough, 1976).

Two basic mechanisms of virus-induced cell fusion have been proposed (Poste, 1970, 1972; Hosaka and Shimizu, 1977; Knutton, 1978). The cell-cell bridge mechanism proposed by Bachi and Howe (1972) and supported by Hosaka and Shimizu (1974, 1977) proposes that virus-specific modifications of the host cell plasmalemma directly promote the fusion of adjacent cells. The alternative cell-viral envelope-cell bridge theory postulates that cell fusion occurs after apposed cells have themselves fused with the same virus envelope and a cytoplasmic bridge is formed. Knutton (1978) has summarized the supporting evidence in each case.

Although herpesviruses and paramyxoviruses are both syncytiogenic, they can be differentiated from each other based on their distinct morphologic and cytologic characteristics. The paramyxoviridae are a family of enveloped RNA-containing animal viruses composed of three genera; paramyxovirus, pneumovirus and morbillivirus (Fenner, 1976). The pneumoviruses including the human and bovine respiratory syncytial viruses (RSV, BRSV) are easily differentiated from the other two genera based on morphological criteria alone. Pneumovirus nucleocapsids are 11-15 nm in diameter while

those of the other genera measure 17-20 nm (Raine et al., 1969; Norrby et al., 1970; McLean and Doan, 1971).

Based on biological, biochemical and antigenic similarities, the morbillivirus genus has traditionally been composed of measles, canine-distemper and rinderpest viruses (Warren, 1960; Imagawa, 1968). Recent evidence suggests that the human subacute sclerosing panencephalitis (SSPE) virus, the virus of the stomatitis pneumoenteritis complex (SPC) and the "Peste des Petits Ruminants" should also be included within the genus (Gibbs et al., 1979; USDA, 1978; Hamdy et al., 1975; Morgan and Rapp, 1977). Morbilliviruses are differentiated from members of the genus paramyxovirus in several ways. The morbilliviruses do not cross react antigenically with other genera, lack a neuraminidase and many strains do not agglutinate mammalian erythrocytes in isotonic buffer (Morgan and Rapp, 1977; Fraser and Martin, 1978). Morbilliviruses readily establish persistent infections in cells of lymphoid or central nervous system origin. Several cell-associated or non-virogenic morbillivirus strains have been recovered using co-cultivation techniques of the virus or chemically induced fusion or infected cells with a susceptible cell line (ter Meulen, 1972a; Fraser and Martin, 1978).

Morbilliviruses and the AMCFV share several biological characteristics with regard to polykaryon formation, lymphotropism, cell-associated and their ability to establish persistent infections in vivo and in vitro. Since the etiology of sheep-associated MCF has not yet been ascertained,

morbilliviruses should not be excluded from candidacy until all MCF isolates have been thoroughly characterized and their disease potential evaluated.

PART II

Identification of a Cell-Associated Morbillivirus from Cattle Affected with MCF: Antigenic Differentiation and Cytologic Characterization

Introduction

Malignant catarrhal fever (MCF) is a highly fatal disease affecting cattle and various other ruminants throughout the world. In the United States, it occurs sporadically, although several epizootics have been reported (Roderick, 1958; Murray and Blood, 1961; Plowright, 1968; Pierson et al., 1973a; Orsborn et al., 1977; Ruth et al., 1977). In Africa, a cell-associated and syncytiogenic herpesvirus, isolated from the blue wildebeest, produced typical MCF when inoculated into cattle (Plowright et al., 1960, 1965a). Cell-associated virus isolation techniques were applied to specimens from MCF-affected cattle in Northern Ireland and Colorado (Clarke et al., 1973; Storz et al., 1976). Numerous isolations of cell-associated polykaryon-forming viruses were made, but their etiologic role with respect to MCF remains unsettled. An isolate from MCF-affected cattle in Northern Ireland was identified as a bovine syncytial virus (BSV). A virus isolated from leukocytes of calf 72-P-535 represents one of 22 Colorado isolates. It was found to be enveloped, cell-associated and syncytiogenic but differed antigenically from BSV (Storz et al., 1976).

In Germany, Bachmann and colleagues (1975) recently isolated a highly cell-associated and syncytiogenic paramyxovirus (V-107) from cattle with sporadic meningoencephalitis. Both polykaryon formation and association with cells are characteristics of the recently isolated bovine leukemia virus (BLV) and cell-associated techniques were required to

isolate a Visna-like virus (BVV) from American cattle (Cornefert-Jensen et al., 1969; Miller et al., 1969; Van der Maaten et al., 1972).

The increasing number of isolations of syncytiogenic and cell-associated viruses from cattle emphasizes the need for developing and improving methods for their identification and differentiation especially where multiple mixed infections are suspected. In this report, isolates from experimental calf #72-P-535 are identified and compared antigenically. The host cell range and dynamics of polykaryon development in infected bovine cells are described.

Materials and Methods

Cell Cultures Used. Bovine fetal spleen (BFS), bovine fetal kidney (BFK), bovine fetal adrenal (BFAdr), bovine fetal thyroid (BFThyr), bovine fetal lung (BFL) and bovine fetal testis (BFTes) cell cultures were prepared using methods previously described (Malmquist et al., 1969; Storz et al., 1976). Cultures were grown at 37 C using Earle's base with lactalbumin-vitamin (Lavit) or Eagle's MEM medium containing streptomycin (500 µg/ml), penicillin (500 units/ml) and 10% lamb serum unless otherwise described.

Virus Strains Used. Isolates investigated were recovered from leukocytes, lymph nodes, thyroid and adrenal glands, spleen and kidneys of experimental MCF calf 72-P-535 (Storz et al., 1976). Virus strain 70-P-1096 was used as the reference strain of bovine parainfluenza type 3 (PI_3).

It was recovered from a calf with pneumoenteritis and is syncytiogenic. Virus isolate 76-R-911 was isolated from leukocytes and other samples from a cow used in the California MCF transmission study described in part V. It was identified antigenically and ultrastructurally as a bovine syncytial virus (BSV) (Malmquist et al., 1969). Isolate 77-R-941 was recovered from the adrenal gland of a calf used in a later passage of the California MCF agent.

Methods Used to Propagate Cell-Associated Virus. Infected monolayers were washed with Saline A prior to being dispersed with trypsin-EDTA and centrifuged at 1750 RPM for 15 min. The cell pellet was resuspended in a small volume of Lavit medium containing 2% heat inactivated lamb serum and added to freshly washed monolayers. The 72-P-535 isolates were propagated in confluent, contact-inhibited monolayers. Strains 76-R-911 and 77-R-941 were propagated in actively growing monolayers.

Test for Cell-Associated Properties and Infectivity of Viral Strains. At the 5th, 10th, 22nd and 49th passage levels of the 72-P-535 (leukocyte) isolate, the cell fraction, cell free culture fluids and infected cells disrupted by freeze-thawing or sonication were tested for infectivity. The culture fluids and cellular components of dispersed cell cultures were separated by centrifugation. Cell-free culture fluid was added to freshly washed monolayers. Cell pellets were resuspended in a small volume of Lavit medium containing 2% inactivated lamb serum. One-third of this cell suspension

was inoculated onto monolayers. Another 1/3 was alternately frozen at -20 C and then quickly thawed at 37 C three times before being used as inoculum. The remaining 1/3 was sonicated at 100 watts for 45 seconds using a Branson S-75 Sonifier Cell Disruptor (Heat Systems Co., Melville, NY) before being added to monolayers. Monolayers were observed daily. The presence and growth characteristics of polykaryons were recorded.

Cytologic Techniques. Confluent monolayers of BFS, BRAdr, BFK, BRThy, BFL and BFTes were grown in Petri dishes containing glass coverslips. A suspension of infected BFS cells in Lavit medium containing 2% inactivated lamb or fetal calf serum (Kansas City Biologicals, Lenexa, KS) was used as inoculum. Coverslips were withdrawn at 12-hour intervals from 12 to 106 hours after inoculation. Each coverslip was rinsed in Dulbecco's PBS (1954), pH 7.4, fixed for 20 minutes in Bouin's fluid and stained by the Giemsa method.

Host Cell Range and Growth Kinetics of Polykaryons. The host cell range and growth kinetics of polykaryons were investigated using the 72-P-535 leukocyte isolate and coverslip preparations of selected primary and continuous cell lines. Primary cell lines tested were as described above. Continuous cell lines including HeLa, MK-2 (Rhesus monkey kidney), VERO (Green monkey kidney) and MDBK (bovine kidney) cells were also investigated.

Infected BFS monolayers were maintained with Lavit medium as previously described. Infected BFK, BFTes, and

BFL monolayers were maintained in Eagle's MEM (Earle's base) with the same concentrations of antibiotics and lamb serum as with BFS. Only infected BFThyr and BFAdr monolayers were maintained with a combination of MEM, antibiotics as described and 2% fetal calf serum (Kansas City Biologicals, Lenexa, KS).

Mouse L-cells (L-929) were grown in Eagle's MEM (Earle's base) supplemented with 10% newborn calf serum (Kansas City Biologicals, Lenexa, KS), streptomycin sulfate (500 μg/ml) and vancomycin (75 μg/ml). Other continuous cell lines were maintained in M-199H with 10% FCS and 1% Pen-Strep-Fungizone (Microbiological Associates, Bethesda, MD).

Monolayers were grown to confluency in plastic Petri dishes containing glass coverslips. Uninfected monolayers were inoculated with a suspension of 72-P-535-infected cells as described above.

At 24, 48, and 72 hours after infection, six coverslips were removed, rinsed in Dulbecco's saline, fixed in Bouin's and stained by the Giemsa method. In addition to the general cytological characteristics of polykaryons, three further observations were recorded: (1) the total number of polykaryons detected in 75 randomly selected microscope fields, (2) the number of fields in which polykaryons were detected, (3) the total number of nuclei observed within polykaryons. From these observations, the average size and relative density of polykaryons/mm^2 was computed for each time point.

Preparation of Antigens for Immunofluorescence Analysis. Monolayers of BFS cells were grown in Petri dishes containing glass coverslips as described above and infected with the 72-P-535, 76-R-911 or 77-R-941 isolates. Coverslips were withdrawn when numerous polykaryons were present.

Monolayers to be infected with PI_3 strain 70-P-1096 were washed with Dulbecco's PBS and the virus was allowed to adsorb for 1 hour at 37 C. When the majority of the cells in parallel cultures adsorbed bovine erythrocytes, coverslips were withdrawn. All cell preparations used in immunofluorescence studies were fixed in cold 4% paraformaldehyde (Eastman Kodak Co., Rochester, NY) containing 0.11 M sucrose and 0.45 mM $CaCl_2$ in PBS, pH 7.2, (Cardiff et al., 1973). Fixed coverslips were rinsed 3 times in PBS, pH 7.7, air dried and stored at -20 C.

Antisera Employed. Antisera against 72-P-535 were produced by inoculating a cow (cow 28) with approximately 10^7 cultured bovine cells infected with 72-P-535 isolates. The animal experienced a transient pyrexia but otherwise appeared normal. The rinderpest antiserum used was prepared at the Plum Island Animal Disease Center in colostrum-deprived calves. A 1:10 dilution neutralized 10^6 TCID/50 of the homologous Kabete "O" strain of virus (Dardiri, A. H.: Personal Communication). The human measles antiserum employed had a neutralization titer of 512 (Breschkin, Alan M.: Personal Communication). Other antisera tested were against canine-distemper virus (CDV), BSV, and bovine respiratory syncytial virus (BRSV).

Fluorescent Antibody Test Procedures. The indirect fluorescent antibody (IFA) test was employed with all antisera except the CDV antiserum which was directly conjugated. Coverslips containing infected cells were thawed, rinsed once in absolute methanol, 3 times in PBS, pH 7.7, and then air dried. Cells were covered with the appropriate dilutions of different antisera and incubated for one hour at 37 C in a moist chamber. Coverslips were removed, given 4 five-minute PBS rinses and air dried. In the indirect tests, coverslips were flooded with a 1:16 dilution of a fluorescein isothiocyanate (FITC)-labeled antiserum prepared in rabbits against bovine IgG (Research Products, Miles Laboratories, Elkhart, IN) or human IgG + IgM + IgA (Cappel Laboratories, Cochranville, PA). Coverslips were then incubated at 37 C for 30 minutes in a humid chamber, rinsed, air dried and mounted in buffered glycerol. Uninfected BFS cells were used as controls.

Fluorescence and Bright Field Microscopy. Fluorescent antibody preparations were viewed using a Leitz Orthomat microscope with an HBO, 200 watt, mercury lamb. The BG-38 (heat absorbing), BG-12 (exciter), and K-530 (barrier) filters were used. Antiserum titers reported are the highest antiserum dilution with which specific fluorescence was observed. Photomicrographs were taken using Kodak EHB-36 color film (ASA 325). Bright field micrographs were made using either the color film described above or Kodak Pan-X (ASA 25). The

same Leitz microscope was employed using neutral density and didydium filters when required.

Neutralization of Polykaryon-Forming Activity in Cell Cultures. Monolayers were infected with isolates 76-R-911, 77-R-941 or 72-P-535 (leukocyte) as described above. However, lamb serum was omitted and 10% antiserum against BSV, 72-P-535, rinderpest virus, CDV or measles virus was substituted. Twenty-four hours after inoculation, infected monolayers were rinsed in Dulbecco's PBS and fresh media containing 10% of the same test antiserum was added. Monolayers were monitored daily and the development of polykaryons was recorded.

Freezing to Maintain Viral Infectivity. Infected cells were resuspended in MEM or Lavit medium. Glycerol (10%) or 7% DMSO plus 15% heat inactivated lamb serum was then added. Ampoules were stored at -70 C or in liquid nitrogen. At intervals, ampoules were removed, cells were thawed rapidly, washed in PBS, and used to inoculate BFS monolayers. Polykaryon formation was monitored daily.

Hemagglutination and Hemadsorption Tests. Cell-free culture fluids and disrupted infected cells were examined for hemagglutinins using the microtiter method. Suspensions (0.25%) of human "O", bovine, and guinea pig erythrocytes were made in PBS, pH 7.2, containing 1% heat-inactivated guinea pig serum. Identical plates were incubated for 2 hours at 4, 22, and 37 C prior to initial reading. Plates were stored overnight at 4 C and reread the next morning.

Hemadsorption procedures were performed on infected and uninfected monolayers grown on coverslips as described. A 0.5% suspension of the same erythrocytes used above, without guinea pig serum, was applied. Incubation was for 1 hour at 22 C. Culture fluids or monolayers infected with PI_3 strain 70-P-1096 were used as a positive control in both tests.

Results

Cytologic Studies. Polykaryons and 72-P-535-infected cells did not fuse with or recruit nuclei from any of the continuous cell lines investigated. Infection was successful and new polykaryons were induced in low passage BFS, BFK, BFThyr, BFADr, BFL and BFTes monolayers. Cytological characteristics were the same regardless of the cell type employed. The 72-P-535 leukocyte isolate grown in BFS monolayers culture is representative and will be described in detail.

Uninfected Lavit-grown BFS cells were epithelioid and contained large ovoid nuclei with multiple irregularly-shaped nucleoli. The cytoplasm was thin, finely granular and weakly basophilic. Mitotic figures were rare in contact-inhibited monolayers, as were cells with 2 to 4 nuclei.

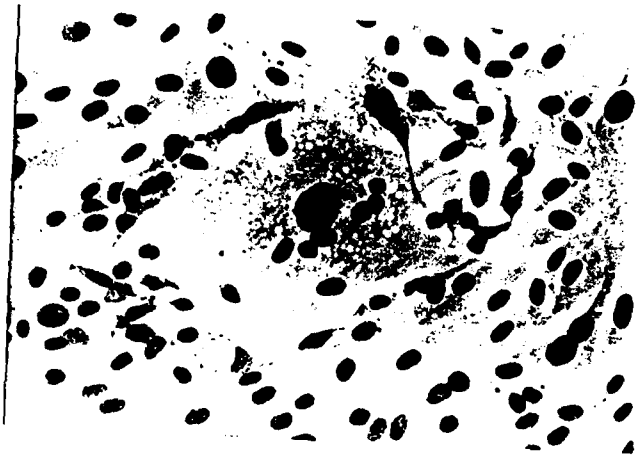
Nuclei identical in size and staining properties to those of uninfected cells were randomly arranged in central or peripheral areas of polykaryons. Neither nuclear nor cytoplasmic inclusions were observed during the course of infection although occasional faintly eosinophilic areas associated with focal cytoplasmic degradation were detected in polykaryons and uninfected cells.

The number of polykaryons depended directly on the number of infected cells in the inoculum. Polykaryon size varied directly with time after inoculation and the degree of secondary fusion. Polykaryons with 5 to 7 nuclei, first detected 24 to 36 hours after inoculation, gradually increased in size and number for the next 12 to 24 hours. Between 48 and 72 hours after infection, cells at the periphery of polykaryons rounded and cytoplasmic basophilia increased (Figures 1,2). In BFS monolayers, large polykaryons containing 150 or more nuclei were produced by the secondary fusion of small polykaryons. Secondary fusion occurred most frequently in BFK monolayers where polykaryons containing 500 nuclei were produced. Between 72 and 84 hours after infection, although polykaryons continued to recruit cells, degenerative changes appeared. Some nuclei stained darkly or became pyknotic and the cytoplasm became increasingly vacuolated (Figure 3). Eventually, polykaryons retracted and detached from the substrate.

In BFS monolayers, the BSV strain, 76-R-911, produced smaller polykaryons containing 12 to 16 nuclei. The nuclei formed a peripheral rosette rather than a central random assemblage (Figure 4). Polykaryons of two distinct morphologies were observed in 77-R-941 infected monolayers. One type was small and similar to those produced by 76-R-911. The other type was large and similar to polykaryons produced by 72-P-535.

Figure 1. Polykaryons produced 48 hours after infection by 72-P-535 leukocyte isolate. Rounded cells were produced at periphery of polykaryon. Cytoplasm had begun to vacuolate. Giemsa stain of BFS monolayer; 250 X.

Figure 2. Giemsa stain of BFS monolayer 60 hours after infection with 72-P-535 leukocyte isolate. Rounded cells were present at periphery of polykaryons. Cytoplasm continued to vacuolate. This monolayer was used in the hemadsorption test. No erythrocytes were adsorbed to polykaryons or other cells; 350 X.



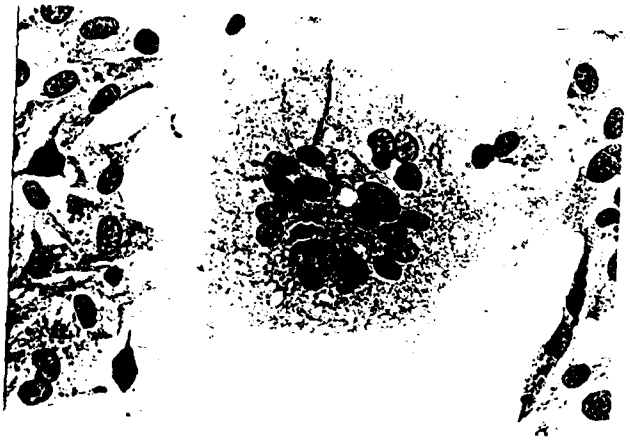
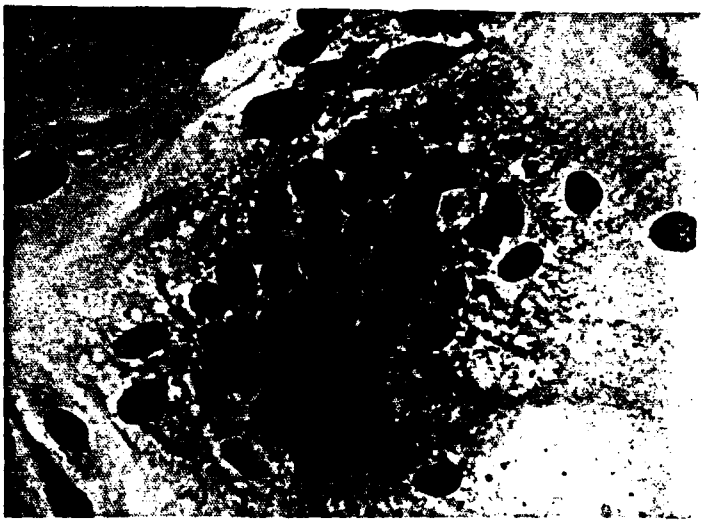


Figure 3. Polykaryon produced 72 hours after infection by 72-P-535 leukocyte isolate. Cytoplasm was intensely vacuolated and some nuclei stained darkly or had degenerated. Giemsa stain of BFS monolayer; 550 X.

Figure 4. Polykaryon produced 60 hours after infection by California MCF isolate 76-R-911 leukocyte isolate. Nuclei were characteristically arranged in a peripheral rosette rather than a central random assemblage. Giemsa stain of BFS monolayer; 400 X.



Host Cell Range and Growth Kinetics of Polykaryons. The computed sizes and densities of 72-P-535 polykaryons grown in four primary bovine fetal cell types are compared in Figures 5 and 6. Only BFS, BFK, BRThyr and BFAdr cells were compared since BFL and BFTes cells did not remain viable through numerous subpassages and thus were not used to isolate cell-associated viruses by co-cultivation.

Polykaryons were most dense in infected BFAdr monolayers while polykaryons with the largest average size were produced in BFThyr monolayers. The increase in size and concomitant decrease in density of polykaryons in BFK monolayers was due to the secondary fusion of polykaryons common with this cell type. Interestingly, BFS monolayers, the cell type with which most MCF isolations have been accomplished, grew to the lowest density and the smallest average size.

Antigenic Differentiation of Isolate 72-P-535 by Immunofluorescence. The titer of homologous (cow 28) serum was 1/32 when tested against the leukocyte, spleen, kidney, lymph node, adrenal, or thyroid isolates from calf 72-P-535. The antiserum to rinderpest virus equalled this titer when tested against cells infected with the 72-P-535 leukocyte isolate (Table 3). Antiserum to measles virus reacted strongly with 72-P-535 antigens at dilutions of 1:4 or less. Canine distemper virus antiserum reacted with 72-P-535 antigens only at a dilution of 1:2.

Antiserum to PI_3 reacted with homologous (70-P-1096) antigens but fluorescence was not seen when 72-P-535 antigens

Figure 5. Size comparison of polykaryons produced by 72-P-535 leukocyte isolate at various times after infection of selected bovine fetal cell cultures.

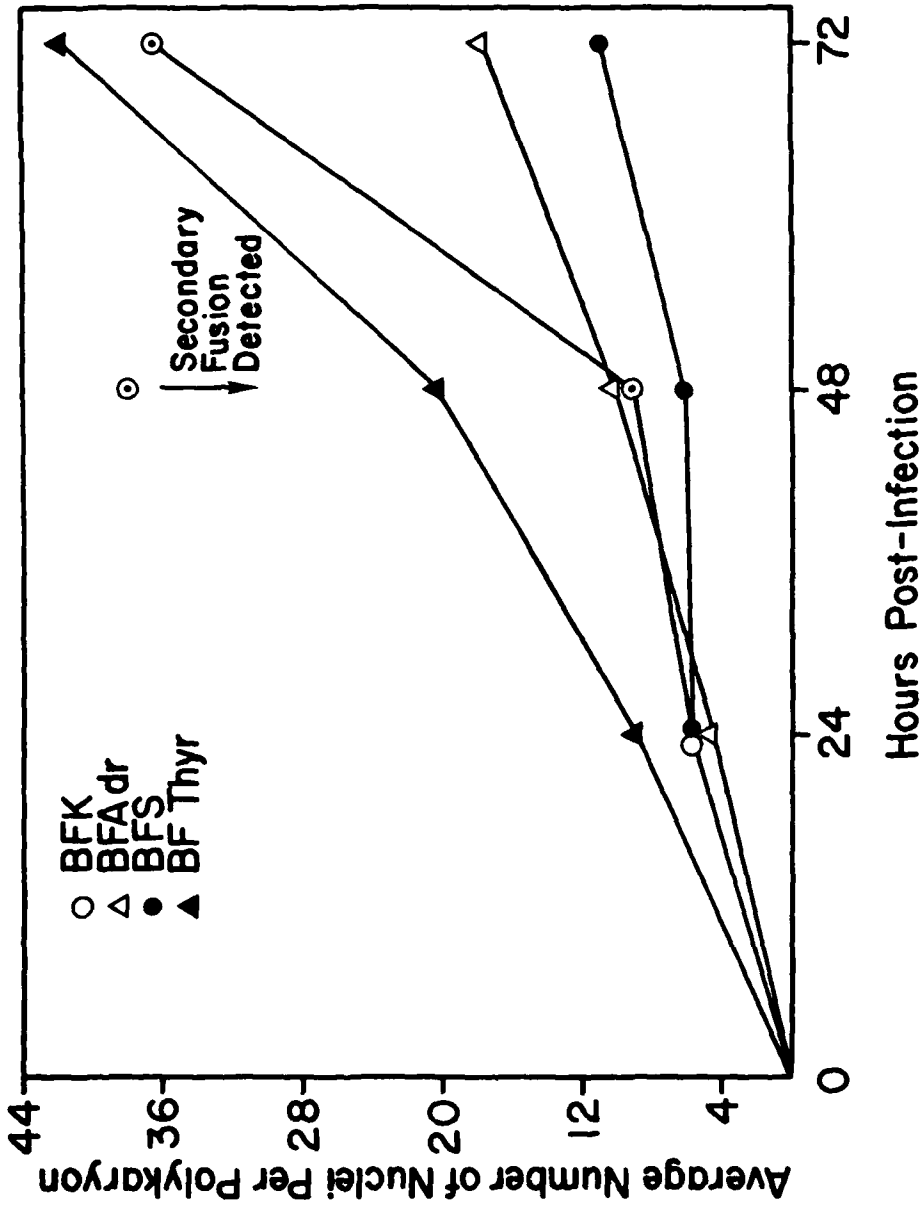
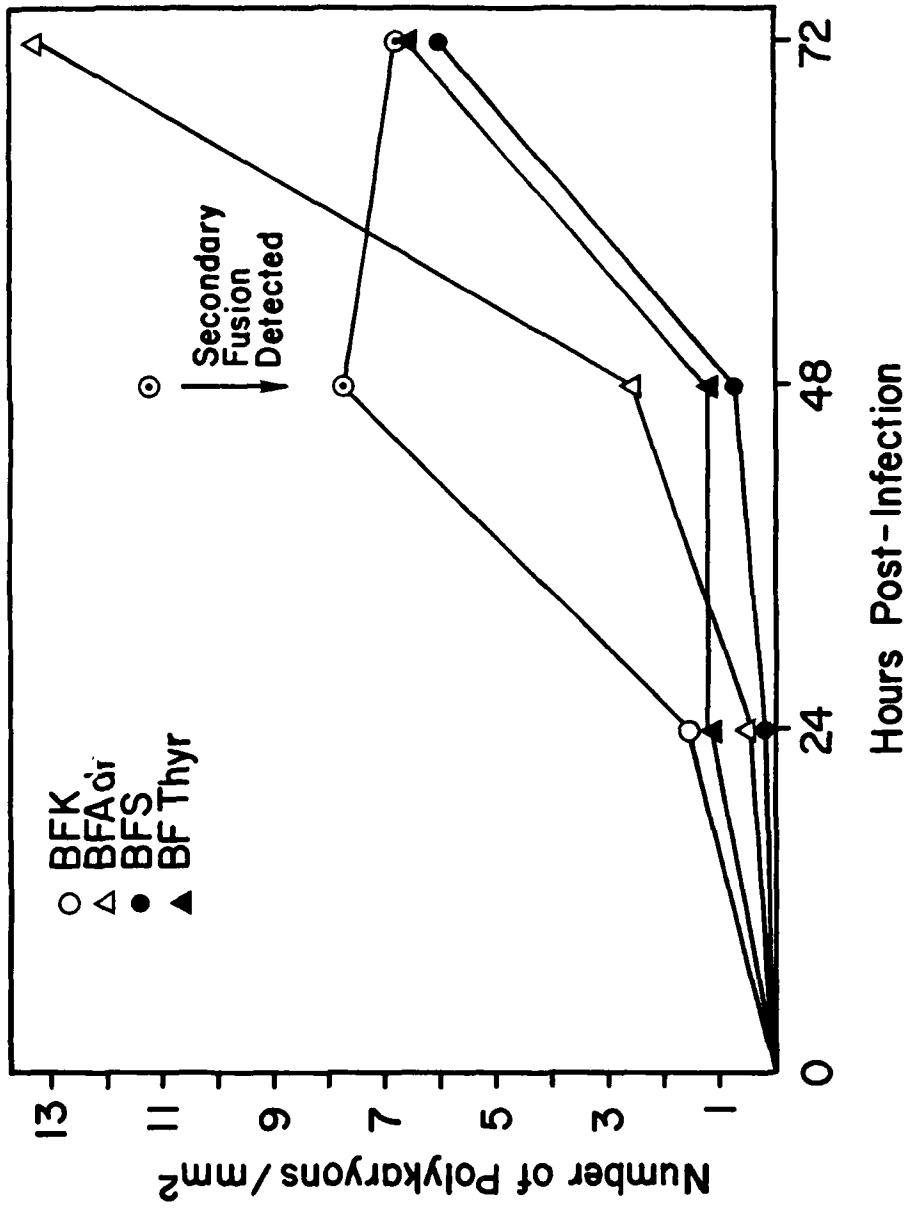

Average Number of Nuclei Per Polykaryon
44
36
28
20
12
4
0
24
48
72
Hours Post-Infection
BFK
BFAdr
BFS
BF Thyr
Secondary Fusion Detected

Figure 6. Comparative densities of polykaryons induced in selected 72-P-535 (leukocyte isolate) infected bovine fetal cell cultures.


Number of Polykaryons / mm²
13
11
9
7
5
3
1
0
24
48
72
Hours Post-Infection
BFK
BFA dr
BFS
BF Thyr
Secondary Fusion Detected

were used. When BSV antiserum was used in a 2-way test, it titered 1/32 when reacted with homologous (76-R-911) antigens (Figure 7). Fluorescence was not seen in tests using BSV antiserum and 72-P-535 antigens. Antiserum to BRSV did not react with PI_3, BSV or 72-P-535 antigens.

Patterns of Fluorescence. In tests employing antiserum from cow 28, the fluorescent patterns observed with each of the 72-P-535 isolates were indistinguishable. Finely granular fluorescence was first confined to the nuclear membrane and perinuclear cytoplasm of small polykaryons and infected peripheral cells. As polykaryons increased in size, fluorescence became more coarsely granular, spread toward the cell boundary and eventually occupied the entire cytoplasm. The centrally located paranuclear cytoplasm fluoresced strongly and occasional large ovoid areas or contiguous lakes of antigen were observed. A weaker fluorescence was observed within the peripheral cytoplasm and associated plasmalemma (Figure 8). Nuclei within 72-P-535 induced polykaryons did not fluoresce.

When CDV, measles or rinderpest antisera were reacted with 72-P-535 polykaryons the cytoplasmic fluorescence pattern was similar to the pattern described with cow 28 serum. However, with the rinderpest and measles virus antisera. small foci of nuclear fluorescence were observed in older polykaryons (Figures 9,10).

Neutralization of Polykaryon-Forming Activity. Antiserum to BSV temporarily inhibited the formation of both

Figure 7. Fluorescence pattern produced when BSV antiserum was reacted with 72-R-911-infected BFS cells. Cytoplasmic fluorescence predominated although transient nuclear fluorescence was observed. 72 hours after infection; 550 X.

Figure 8. Results of indirect FA test using cow 28 serum and 72-P-535 leukocyte isolate. Diffuse granular fluorescence of the paranuclear cytoplasm was evident. Nuclei did not fluoresce. All 72-P-535 isolates produced the same fluorescence pattern. BFS monolayer, 84 hours after infection; 550 X.

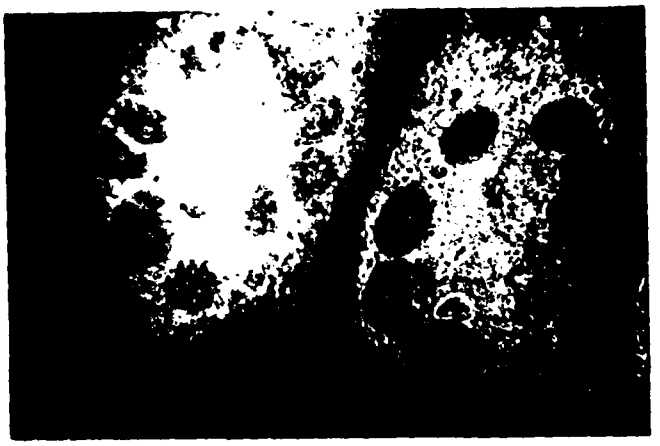
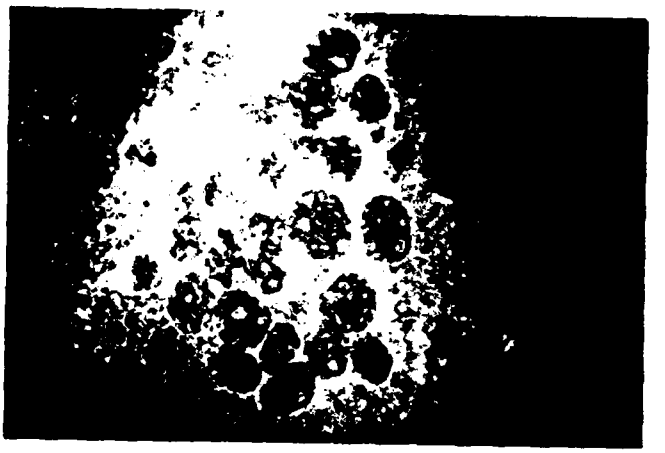


Figure 9. Fluorescence pattern observed when rinderpest virus antiserum was reacted with 72-P-535 polykaryons. Diffuse fluorescence was detected throughout the cytoplasm and within some nuclei. BFS monolayer, 84 hours after infection; 550 X.

Figure 10. Results of immunofluorescence test using measles virus antiserum and 72-P-535-infected monolayer. Fluorescence was initially confined to the nuclear membrane and perinuclear cytoplasm. Both cytoplasmic and nuclear fluorescence was observed. BFS monolayer, 84 hours after infection; 550 X.



76-R-911 and the small 77-R-941 polykaryons. When infected monolayers were subsequently passed in the presence of inactivated lamb serum, polykaryons reappeared in each case. Serum from cow 28 completely inhibited the formation of 72-P-535 polykaryons. Polykaryons developed within several days if the culture medium were changed and lamb serum was added. When 77-R-941 was passed using media containing 10% cow 28 and BSV antiserums, the small polykaryons were suppressed completely. Although there was a large reduction in the number of large polykaryons, a few did develop.

Antiserums to measles and CDV completely inhibited formation of 72-P-535 polykaryons. Rinderpest virus antiserum reduced their number by 75%.

Cell-Associated Properties and Preservation of Infectivity. In 72-P-535 infected monolayers, infectivity resided exclusively within the cellular fraction through 49 consecutive passages. Cell-free supernatant fluids, cell sonicates and lysates were not infectious (Table 4). Freezing in liquid nitrogen using Lavit medium, 7% DMSO and 15% inactivated lamb serum was the superior regimen for the maintenance of viral infectivity. Using this procedure all isolates regain prefreezing viability within two passages.

Hemagglutination and Hemadsorption Tests. Neither supernatant fluids nor lysates of 72-P-535 infected cells agglutinated guinea pig, human "O" or bovine erythrocytes.

Numerous bovine erythrocytes adsorbed to polykaryons and single cells in monolayers infected with PI_3 strain

Table 3. Results of Immunofluorescence Studies Using Virus 72-P-535 (Leukocyte Isolate) and Selected Antiserums

Antigens	Antiserums						
	Cow 28	Rinderpest	Measles	Distemper	PI_3	BSV	BRSV
72-P-535 (Leukocyte)	$\geq$ 1:32	$\geq$ 1:32	1:4	1:2	< 1:8*	< 1:8*	< 1:8*
70-P-1096 (PI_3)	< 1:4*	< 1:4*	< 1:2*	NEG	1:16	< 1:8*	< 1:8*
77-R-911 (BSV)	< 1:2*	< 1:2*	< 1:2*	< 1:2	NEG	> 1:32	> 1:8
Control (BFS)	NEG	NEG	NEG	NEG	NEG	NEG	NEG

NEG = Negative test with undiluted and diluted serums.

* = Lowest dilution tested.

Table 4. Nature of Cell-Associated Infectivity of Virus 72-P-535 (Leukocyte Isolate) at Various Passage Levels

Type of Treatment	Polykaryon-formation in BFS Monolayers at Passage:			
	5	10	22	49
Cell Fraction	+	+	+	+
Tissue Culture Supernatant (2,000 RPM X 10')	-	-	-	-
Freeze-Thawing	-	-	-	-
Sonication	-	-	-	-

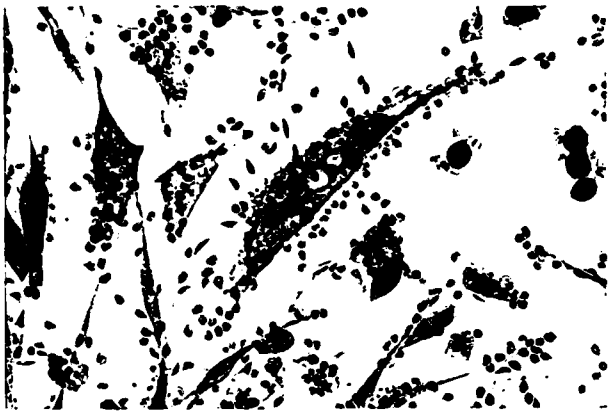
70-P-1096 (Figure 11). In monolayers infected with 72-P-535, bovine erythrocytes did not adsorb to polykaryons or peripheral cells (Figure 2). Uninfected controls were negative in both tests.

Discussion

The paramyxoviridae are a family of viruses which includes the genera paramyxovirus, morbillivirus and pneumovirus (Fenner, 1976). Sendai virus, also called the hemagglutinating virus of Japan (HVJ), Newcastle disease virus (NDV) and parainfluenza types 1 to 5 (PI_{1-5}), are well-known members of the genus paramyxovirus. The morbilliviruses (measles, canine-distemper and rinderpest) are morphologically similar to the paramyxoviruses but differ from them in several respects. Morbilliviruses lack a neuraminidase, share antigens only within the genus and either do not agglutinate erythrocytes or do so only under specific conditions (Örvell and Norrby, 1974; Morgan and Rapp, 1977).

Because of their strong antigenic interrelationship, various serological tests have been used in the diagnosis and laboratory investigation or morbilliviruses (Warren, 1960; Imagawa, 1968). Among these, the fluorescent antibody test has been reported the method of choice for the demonstration of morbillivirus cross-antigenicity as well as for the detection of virus-specific antibody in serum (Yamanouchi et al., 1970). Based on the results of the fluorescent antibody tests, we have demonstrated cross-reactivity between

Figure 11. Bovine erythrocytes absorbed to BFS cells infected with PI_3 strain 70-P-1096. Giemsa stain; 350 X.



isolate 72-P-535 and antiserum against other members of the morbillivirus genus. Additionally, the pattern and development of virus-specific fluorescence within infected cells agrees with patterns reported for morbilliviruses (Yamanouchi et al., 1970). Based on the results of the fluorescent antibody tests and the ability of selected serums to suppress polykaryocytosis within infected monolayers, the viruses recovered from animal 72-P-535 represent multiple isolates of a virus which is antigenically related to measles, canine-distemper and rinderpest viruses.

Despite antigenic and other similarities, morbilliviruses often differ with regard to certain biological, cytological and pathogenic properties. Measles virus is the only morbillivirus for which hemadsorption (HAD) or hemagglutination (HA) is consistently demonstrable (Gorham, 1960; Rosanoff, 1960; Liess and Plowright, 1963). Most measles virus strains will hemadsorb or hemagglutinate simian erythrocytes at physiological pH in isotonic buffer. Some "salt-dependent" measles strains required higher molarity diluents (Breschkin et al., 1977). Both Huygelen (1960) and Plowright (1963) were unable to demonstrate hemagglutination by rinderpest virus although Liess (1966) reported that the Kabete "O" strain agglutinated the erythrocytes of several mammalian species. Hemadsorption by rinderpest infected cells has not been reported (Gorham, 1960; Liess, 1966). There have been conflicting reports concerning the ability of CDV to hemagglutinate, but it is not considered a

characteristic of the virus (Appel and Gillespie, 1971). Cells infected with 72-P-535 isolates did not adsorb nor did lysates or infected fluids agglutinate bovine erythrocytes under conditions required for the same activity by PI_3. Although incubation temperatures were varied and erythrocytes from several species were evaluated, neither hemadsorption nor hemagglutination was ever demonstrated.

Some cytological characteristics of the 72-P-535 leukocyte isolate were reported earlier by Storz and associates (1976). Although no quantitative or qualitative comparisons were reported, they observed that this isolate produced polykaryons in BFS, BFAdr, BFThyr and bighorn sheep thyroid monolayers. Polykaryons were not detected in BFK, MDBK, HeLa or L-cells. Results reported here confirm that 72-P-535 does not produce polykaryons in the continuous cell lines tested but disagree with the earlier findings with regard to BFK monolayers.

The observed differences in polykaryon size and rate of recruitment suggest that these cell types vary in their susceptibility to virus-induced fusion and in the ability of infected cells or fragments of polykaryons to establish new infectious centers. Even though BFS cells were not as susceptible to fusion as the other cell types tested, their viability through the numerous subpassages required in cocultivation techniques warrants their continued use.

The non-infectious nature of cell-free culture fluids, cell lysates and sonicates confirms the earlier report

(Storz et al., 1976) that, even after numerous cell passages, the infectivity of 72-P-535 is strictly cell-associated. High cell-associated to cell-released virus ratios are typical in cells productively infected and morbilliviruses and released virions are relatively heat labile (Black et al., 1959; Karzon, 1962; Arita and Matumoto, 1968). Morbillivirus variants which are cell-associated upon initial isolation frequently are released from infected cells after repeated tissue culture passages, shift of incubation temperature, fusion with permissive cells or reinoculation into animals (Knight et al., 1972; Haspel et al., 1973; Lampert et al., 1976). The factors contributing to the cell-associated nature of 72-P-535 require further investigation with respect to the possible roles of defective particles, temperature sensitivity, thermal lability and host cell-induced modifications of released virus.

Differences in polykaryon morphology and the selective antiserum-mediated suppression of polykaryon formation indicate that MCF isolate 77-R-941 represented a multiple mixed infection by two cell-associated, syncytiogenic viruses. One isolate is apparently BSV while the other is related antigenically to 72-P-535. Bovine syncytial virus is ubiquitous within the cattle populations of the world (Scott et al., 1973) and has previously been isolated from MCF cattle Clarke et al., 1973). When present in multiple mixed infections, BSV is known to interfere with the isolation of other syncytiogenic viruses present (Van der Maaten et al., 1972).

Storz and coworkers observed that multiple-mixed infections of cattle by cell-associated viruses may go unrecognized unless reliable procedures are developed to overcome difficulties inherent in their isolation and separation (Storz et al., 1976). Immunofluorescence techniques, the selective suppression of polykaryocytosis by antiserums and electron microscopy are valuable techniques in this regard.

Multiple mixed infections involving cell-associated viruses could influence the pathogenesis of a disease by causing the recrudescence of a latent virus or by modulating the susceptibility of the host. Synergistic interactions could result in a condition which is atypical of either virus acting alone.

Morbilliviruses primarily affect lymphoid elements, usually causing lymphocytolysis and destruction of lymphoreticular tissues. Besides Bachmann's morbilli-like V-107 (1975), rinderpest is the only morbillivirus known to affect cattle. In rinderpest and possibly MCF, alterations of lymphoid cells or organs play a critical role in the pathogenesis of the disease. Rinderpest virus has been reported to cause clinically silent infections and many members of the family paramyxoviridae are known for their ability to cause persistent infections in vivo and in vitro (Plowright, 1968; Knight et al., 1972; ter Meulen et al., 1972; Morgan and Rapp, 1977; ter Meulen and Hall, 1978). Both measles and canine-distemper viruses are implicated in persistent infections of the central nervous system (CNS) which may

have autoallergic components (Appel and Gillespie, 1971; Wear and Rapp, 1971; Agnarsdottir, 1977; Morgan and Rapp, 1977; ter Meulen and Hall, 1978).

Recently, Liggitt and colleagues successfully transmitted MCF to calves using whole blood or purified mononuclear cells from an affected animal. Their gross histopathological and ultrastructural findings are compatible with the hypothesis that the widespread lymphoproliferation, vascular changes and epithelial lesions characteristic of MCF may be manifestations of exaggerated lymphoreactivity.

Although rinderpest has not been reported from North American cattle, a cell-associated variant of rinderpest virus could exist in certain subpopulations which, if activated by environmental stresses or the presence of second virus, might lead to a pansystemic lymphoproliferative disease. We believe this hypothesis to be consistent with the known biological characteristics of the morbilliviruses and with diseases known to be caused by its members.

Part III

The Morphogenesis and Cytopathology of a Cell-Associated Bovine Morbillivirus in BFS Cells

Introduction

Malignant catarrhal fever is a pansystemic lymphoproliferative disease of cattle and other ruminants throughout the world (Plowright, 1968). Although Plowright and coworkers (1965a) isolated a syncytiogenic, cell-associated herpesvirus from African wildebeest which produced MCF after inoculation of cattle, the cause of the North American form of MCF is currently elusive. In Colorado, Storz and associates isolated a cytolytic herpesvirus and several syncytiogenic, cell-associated viruses from cattle with MCF (Storz, 1968; and colleagues, 1976). The herpesvirus (66-P-347) was not neutralized by antiserum to the African MCF virus (AMCFV) (Kalunda, 1975; Storz et al., 1976). Cell cultures infected with syncytiogenic isolate 72-P-535 released enveloped particles into the extracellular fluid although infectivity remained strictly cell-associated (Storz et al., 1976; Coulter and Storz, 1979). Using immunofluorescent and other serological techniques, Coulter and Storz (1979) tentatively identified 72-P-535 as a morbillivirus. The recovery of 72-P-535 from leukocytes, reticuloendothelial, lymphoid, adrenal and thyroid cells is consistent with the lymphotropic nature of morbilliviruses and their ability to establish persistent infections in such tissues (Horta-Barbosa et al., 1971; Tajima et al., 1971a; Joseph et al., 1975; Barry et al., 1976). Liggitt and colleagues (1978b) transmitted MCF to cattle using leukocytes or the gradient-separated mononuclear cell fraction of blood. They also observed that a non-lytic

(persistent) virus infection of a lymphocyte sub-population could induce a disease with the immunopathological changes characteristic of malignant catarrhal fever.

The strict association of infectivity with the cell as well as other biological properties of 72-P-535 are similar to other morbillivirus strains implicated in chronic, degenerative, demyelinative diseases of man and other animals with a possible immunopathological basis (ter Meulen et al., 1972b; McCullough et al., 1974; Bachmann et al., 1975; Morgan and Rapp, 1977). Ultrastructural investigations have revealed morphogenic and cytopathogenic differences between morbilliviruses which cause persistent rather than lytic infections (Dubois-Dalcq et al., 1974; Kratzsch et al., 1977; Confer et al., 1975a; Barry et al., 1976).

The observation that the pathogenic potential of morbilliviruses is consistent with their involvement in a disease with the pathological features of MCF was of special interest since no etiological agent had yet been found. Morbilliviruses, members of the measles, canine-distemper, rinderpest virus group, had not previously been isolated from North American cattle and additional support for the serological findings and original classification were required. Ultrastructural features of the morphology, morphogenesis and cytopathology of 72-P-535 grown in bovine fetal spleen cells and maintained using cell-associated techniques were investigated and are described in this report.

Materials and Methods

Propagation of Cell Cultures and Virus Isolates. Monolayers of sixth passage bovine fetal spleen (BFS) cells used in ultrastructural investigations were grown in Petri plates using Earle's base with lactalbumin-vitamin (Lavit) medium containing streptomycin (500 µg/ml) penicillin (500 units/ml) and 10% heat-inactivated lamb serum as described in part II. Heat-inactivated lamb serum was reduced to 2% in infected cell cultures.

The leukocyte isolate of virus 72-P-535 used in this study was propagated in low passage cells as described in part II. Briefly, when infected monolayers contained numerous polykaryons, the culture fluid was decanted and the monolayer dispersed with trypsin-EDTA. The cell fraction was pelleted and then resuspended in Lavit medium supplemented with inactivated lamb serum. Half the cell suspension was added to freshly washed uninfected BFS monolayers. Fluids from infected cultures added to BFS monolayers did not induce polykaryons. Culture fluids tested on supplemented PPLO agar (Hayflick, 1965) and electron microscopic examination of infected cells proved them free of mycoplasma.

Electron Microscopic Investigations of Virus Released from Infected Cells. Culture fluids from infected monolayers were partially clarified and stored at -20 C prior to examination. The fluid was thawed and centrifuged at 74,000 g for 90 minutes at 4 C. The pellet was resuspended in 1.5 ml of 5% sucrose (w/w) in TE buffer (0.005 M Tris-HCl, 0.001 M EDTA;

pH 7.4), layered onto a linear 20-40% (w/w) sucrose gradient and centrifuged at 81,000 g for 2 hours at 4 C. Serial drip fractions were collected and dialyzed at 4 C overnight against a large excess of 1% ammonium acetate then concentrated by dialysis against 33% (w/v) polyethylene glycol (m.w. 20,000) in TE buffer. Samples were applied to formvar-carbon coated grids and negatively stained using 3% aqueous phosphotungstate, pH 7.2.

Preparation of Infected Cells for Ultrastructural Analysis. Infected monolayers were prepared for electron microscopic analysis using a modified in situ fixation and embedding technique (Brinkley et al., 1967). After infection, monolayers were withdrawn at 12-hour intervals and rinsed twice in Dulbecco's saline, pH 7.2. Monolayers were fixed for 1 hour in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.25 M sucrose. Further fixation was accomplished using 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) prior to dehydration through alcohols. Dehydration was completed in hydroxypropyl methacrylate before monolayers were embedded in Epon 812. After polymerization at 60 C for 48 hours, areas to be studied were removed, sectioned with a Porter-Bluhm MT-2B ultratome using a diamond knife and stained with uranyl acetate followed by lead citrate. Thin sections were viewed with an Hitachi HU-12 electron microscope operated at 75 Kv acceleration.

Results

Morphologic Properties of Released Virus Particles. The appearance of a representative cellulose nitrate tube after centrifugation is depicted in Figure 39b. A total of 12 serial drip fractions were obtained and analyzed. Enveloped virus particles 150-500 nm in diameter were scattered throughout the cloudy central portion of the tube. Most particles were round or ovoid and lacked well-defined surface projections. Striated tubular structures identified as viral nucleocapsids were wound inside intact particles. Disrupted particles released nucleocapsids 17-20 nm in diameter with "herring bone" surface striations 5-7 nm apart (Figures 12, 13).

Virus Morphogenesis, Cell Fusion and Ultrastructural Cytopathology.

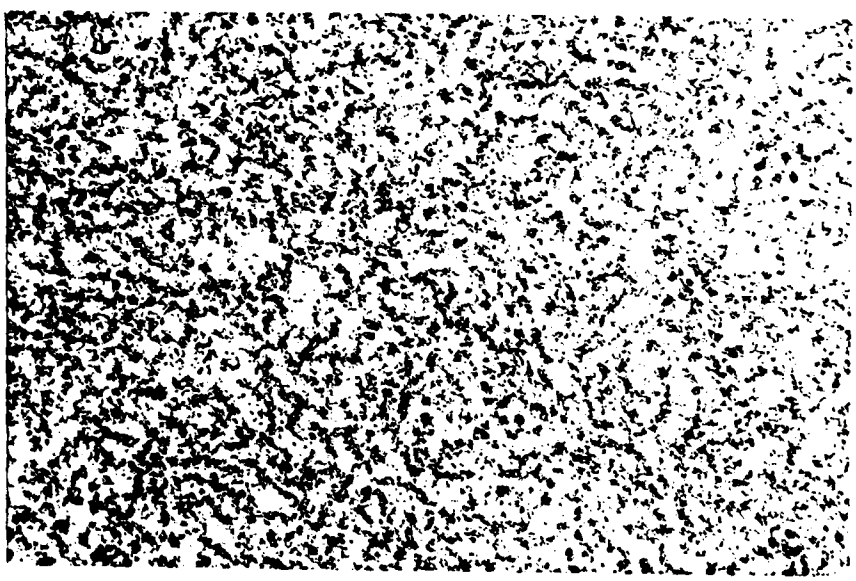
Virus Morphogenesis. Viral structures were not detected in infected cells before 36 hours after infection. The first virus-specific changes were observed within the perinuclear cytoplasm of developing polykaryons between 36 and 48 hours after infection. Except for ribosomes, cellular organelles were displaced from juxtanuclear sites where nucleocapsids developed within a granulofibrillar matrix (Figure 14). Although subunit features were partially obscured, nucleocapsids were identifiable as helical intertwined structures 17-20 nm in diameter. Each contained a central hollow channel and had surface striations with a 5-7 nm periodicity (Figure 15). As infection proceeded,

Figure 12. Nucleocapsids released from disrupted particles recovered from culture fluids of 72-P-535 infected BFS monolayers. Surface striations 5-7 nm apart were visible. PTA stain; 120,000 X.

Figure 13. Higher magnification of nucleocapsids released from disrupted particles. Periodically of surface striations is more clearly visible. PTA stain; 210,000 X.

Figure 14. Cytplasm of BFS cell 48 hours after infection with 72-P-535 leukocyte isolate. Nucleocapsids (arrows) were developing in association with a granulofibrillar material which partially obscured surface details; 20,000 X.

Figure 15. Fuzzy nucleocapsids within the cytoplasm of a 72-P-535-infected BFS cell. Surface striations and 5-7 nm central channel were evident 72 hours after infection; 30,000 X.

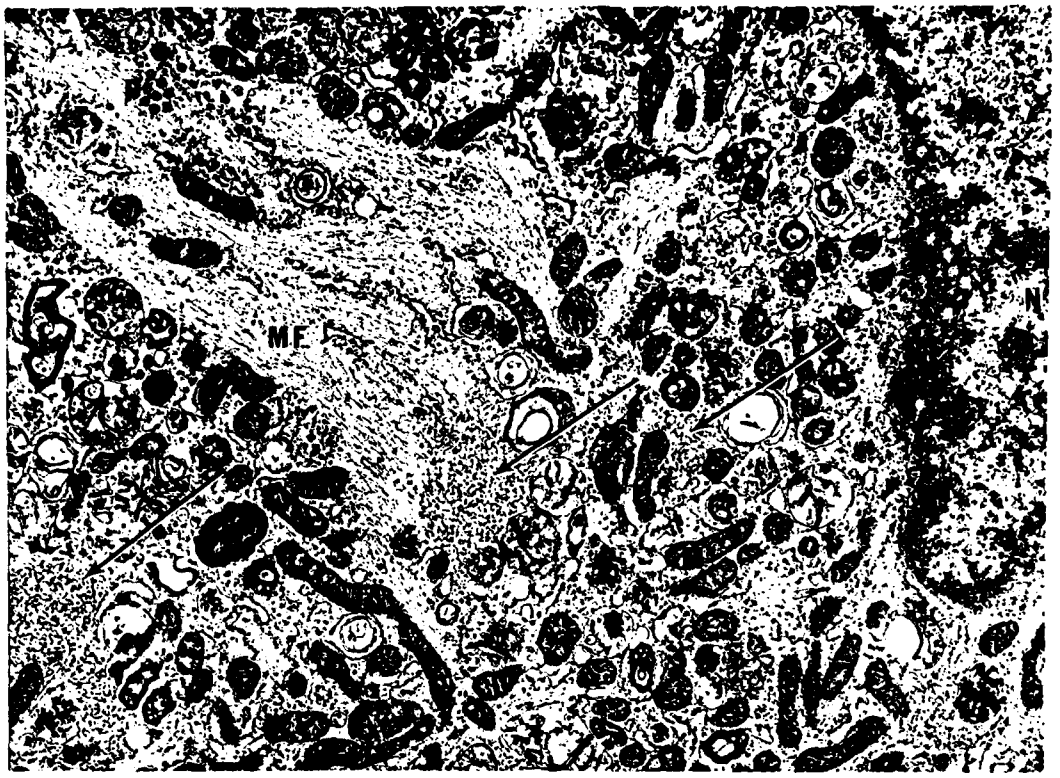


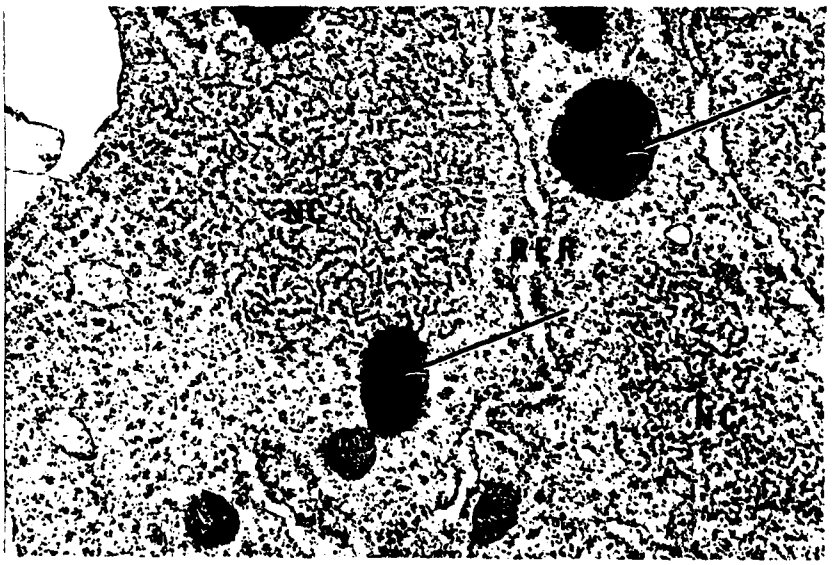
nucleocapsids with associated granulofibrillar material (fuzzy nucleocapsids) increased in number and accumulated in areas throughout the cytoplasm (Figures 16,17). Nucleocapsids were often aligned in a parallel or random manner beneath thickened, osmiophilic regions of plasmalemma containing a hazy external fringe (Figure 18). Late in infection, nucleocapsids were occasionally observed within the nuclei of polykaryons (Figure 19). Intranuclear nucleocapsids were not associated with granulofibrillar material (smooth nucleocapsids) but were otherwise identical to the fuzzy intracytoplasmic forms. Small accumulations of intranuclear smooth nucleocapsids were frequently associated with areas of microfilamentous material comprising "nuclear bodies" Figures 20, 21).

Extracellular virus particles were released when underlying nucleocapsids became enveloped at specialized areas of plasmalemma (Figures 22,23). Small numbers of highly pleomorphic particles 150-500 nm or more in longest dimension were produced (Figures 24,25). Round or ovoid forms predominated although serpentine and other bizarre forms were observed (Figure 26). Early in the course of infection, the periphery of most released particles was covered by a 9-11 nm continuous and hazy figure. In particles produced late in infection, the fringe was often discontinuous, patchy or completely absent (Figure 27). Nucleocapsids could not be detected in some released particles although most contained either fuzzy or smooth nucleocapsids. Particles containing

Figure 16. Small accumulations of nucleocapsids (arrows) developed within the cytoplasm of a BFS cell. Lysosomal structures (L), nucleus (N), microfilaments (MF), mitochondria (M) and polysomes (P) were also visible. 60 hours after infection; 15,000 X.

Figure 17. Collections of fuzzy nucleocapsids (NC) in the cytoplasm of a BFS polykaryon developed 60 hours after infection with the 72-P-535 leukocyte isolate. Rough endoplasmic reticulum (RER) was dilated and lipid droplets (arrows) were evident; 24,000 X.


MF
N


RER

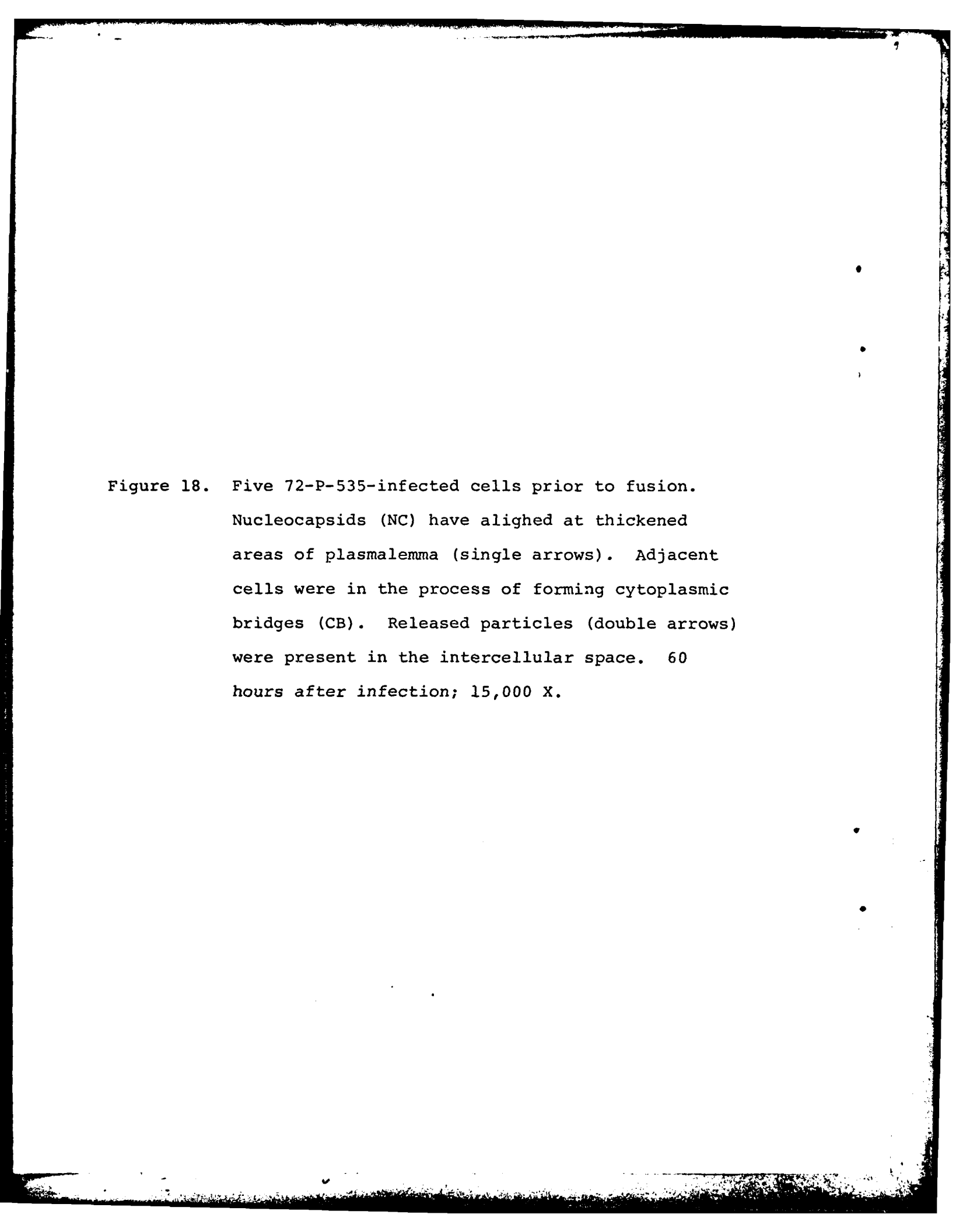


Figure 18. Five 72-P-535-infected cells prior to fusion. Nucleocapsids (NC) have alighed at thickened areas of plasmalemma (single arrows). Adjacent cells were in the process of forming cytoplasmic bridges (CB). Released particles (double arrows) were present in the intercellular space. 60 hours after infection; 15,000 X.

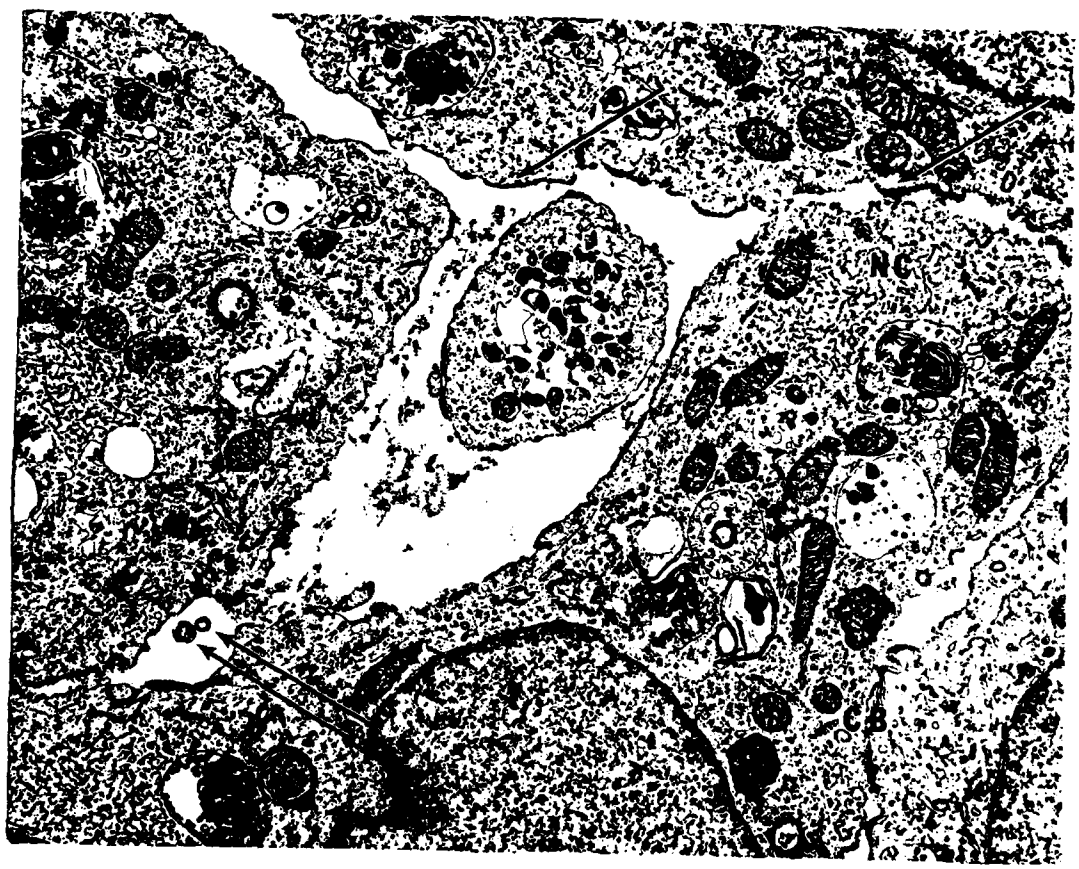
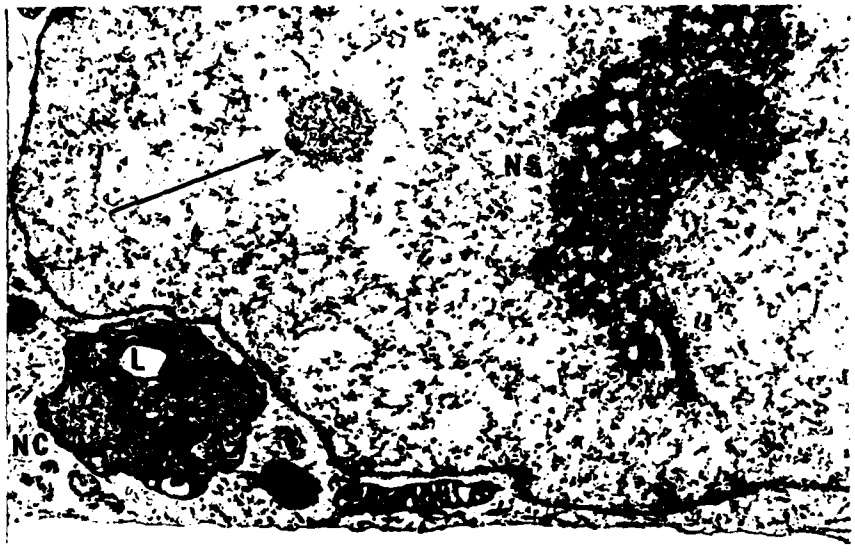

N
C
B

Figure 19. Nuclear body (arrow) within a cleared area of nucleoplasm of a BFS cell. NC = cytoplasmic nucleocapsids; L = secondary lysosome; NS = nucleolus. 106 hours after infection; 12,500 X.

Figure 20. Smooth nucleocapsids associated with microfilamentous material (arrow) constituted a nuclear body. 72-P-535 leukocyte infection of a BFS cell 96 hours after infection; 47,500 X.


NS
L
NC

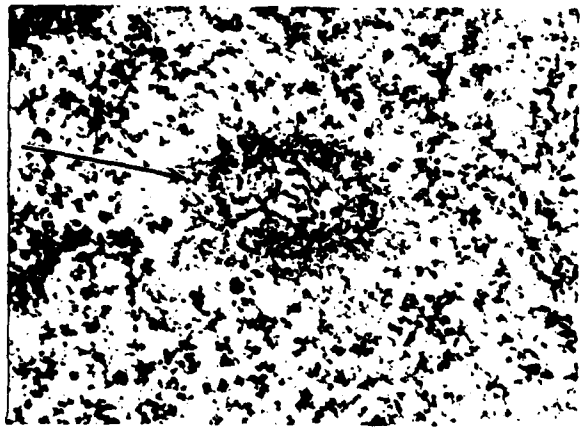


Figure 21. A loosely arranged nuclear body within an infected BFS cell. The close association of nucleocapsids with filamentous material (arrow) was evident. 106 hours after infection with 72-P-535 leukocyte isolate; 37,500 X.

Figure 22. Virus particle released from BFS polykaryon 60 hours after infection with 72-P-535 leukocyte isolate. Morphology was typical of particles released early in infection; 75,000 X.

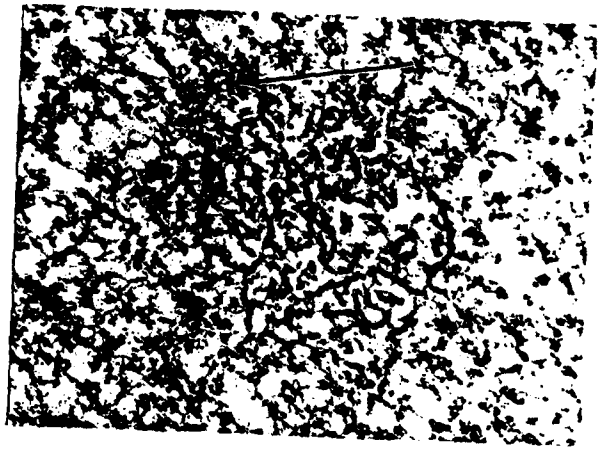


Figure 23. Virus particles released from infected BFS cell 72 hours after infection with 72-P-535 leukocyte isolate. Nucleocapsids were loosely arranged although external fringe appeared intact. NC = cytoplasmic nucleocapsids; 60,000 X.

Figure 24. Pleomorphic enveloped particles budded from the plasmalemma of infected BFS cells. Rounded particles with densely or loosely packed nucleocapsids were common. Some particles had a patchy external finge and contained host cell components (arrows). 72 hours after infection; 37,000 X.

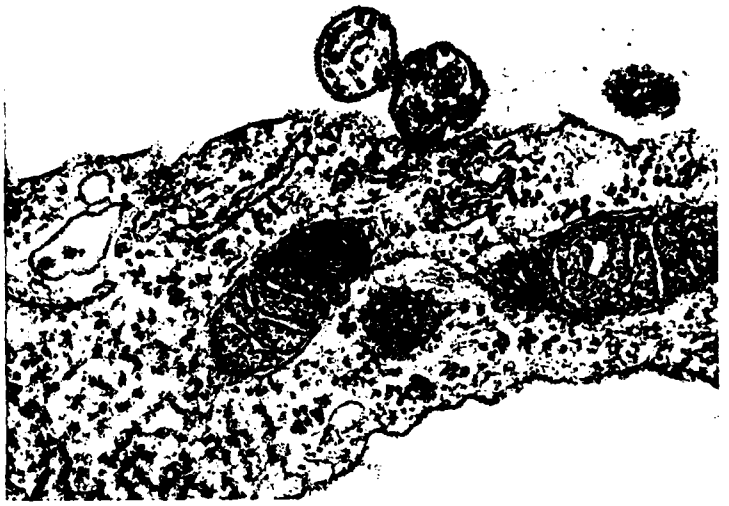


Figure 25. Pleomorphic virus particles released from BFS polykaryon 72 hours after infection with 72-P-535 leukocyte isolate. Most particles were round or ovoid. The hazy external fringe and alignment of nucleocapsids were evident; 50,000 X.

Figure 26. Irregularly shaped particles containing parallel tortuously coiled nucleocapsids were budded from BFS polykaryons late in infection. Such forms were apparently multiploid, possibly the product of an aberrant assembly process. 48 hours after infection; 42,500 X.

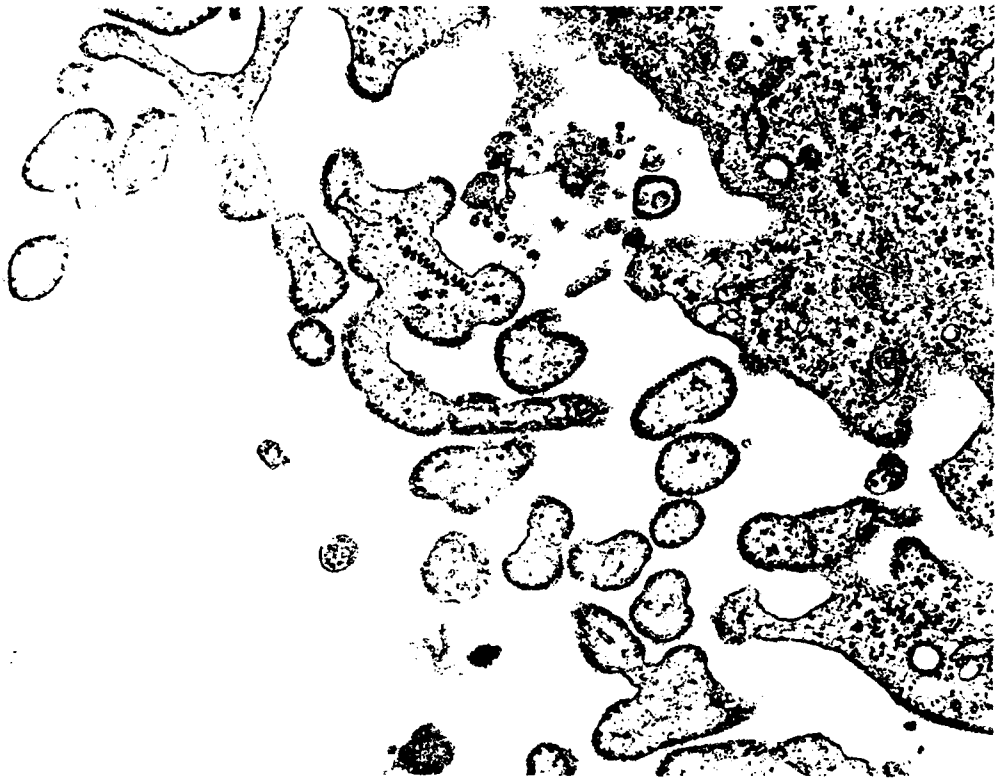
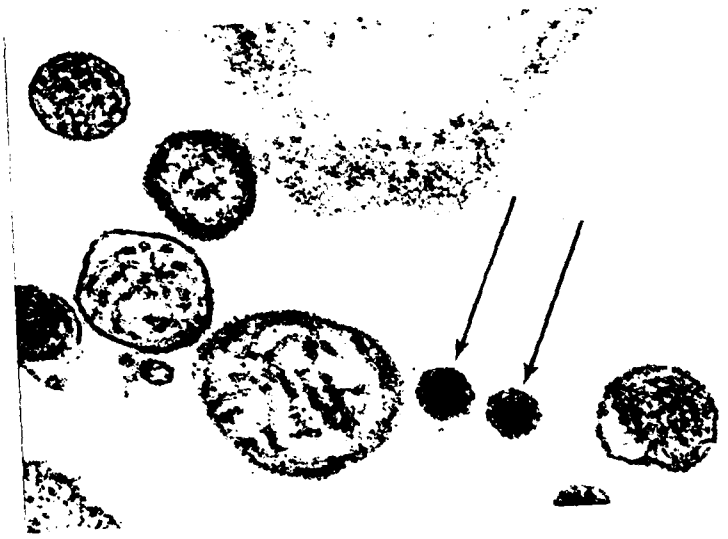


Figure 27. Late in infection, BFS polykaryons released particles which contained loosely packed smooth nucleocapsids. The external fringe was reduced or absent. Some particles appeared normal while others contained a dense granular material (arrows). 72-P-535 leukocyte isolate, 96 hours after infection; 62,500 X.



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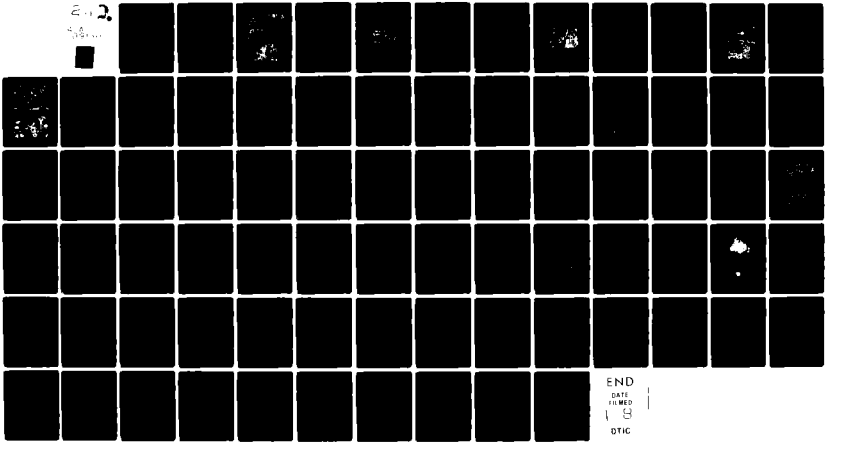

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randomly arranged and loosely packed nucleocapsid and, occasionally, host cell components were more frequently observed late in infection (Figure 27).

Concomitant with the release of virus particles from the cell surface, virus-induced cytoplasmic and nuclear changes were observed within polykaryons. Beginning 72-84 hours after infection, budding activity diminished markedly and large intracytoplasmic accumulations of densely packed nucleocapsids developed. During this period the amount of fibrillar material associated with nucleocapsids became reduced and eventually large accumulations of smooth nucleocapsids were formed. Smooth nucleocapsids were released en masse into the extracellular space upon cytolysis (Figures 28,29).

Ultrastructural Features of Polykaryon Formation. Mitoses within polykaryons were neither observed during this study nor during a previously described cytological investigation (Coulter and Storz, 1979). Virus-induced cell fusion was first detected 36 hours after infection and continued through 130 hours. Large polykaryons developed by the successive recruitment of mononucleated cells or by the secondary fusion of small polykaryons.

Cell fusion was initiated at thickened portions of plasmalemma which were osmiophilic and frequently covered by a hazy 9-11 nm external fringe. Fusion began after these areas evaginated in a process typical of virus budding (Figure 30). Nucleocapsids were observed near such differentiated portions

Figure 28. Large numbers of smooth nucleocapsids were released en masse into the extracellular space after cytolysis of infected BFS cells. 106 hours after infection; 51,000 X.

Figure 29. Higher magnification of smooth nucleocapsids released from cytoplasm of infected BFS cell. Surface striations and central hollow channel were clearly visible due to lack of associated granulofibrillar material. 96 hours after infection; 100,000 X.

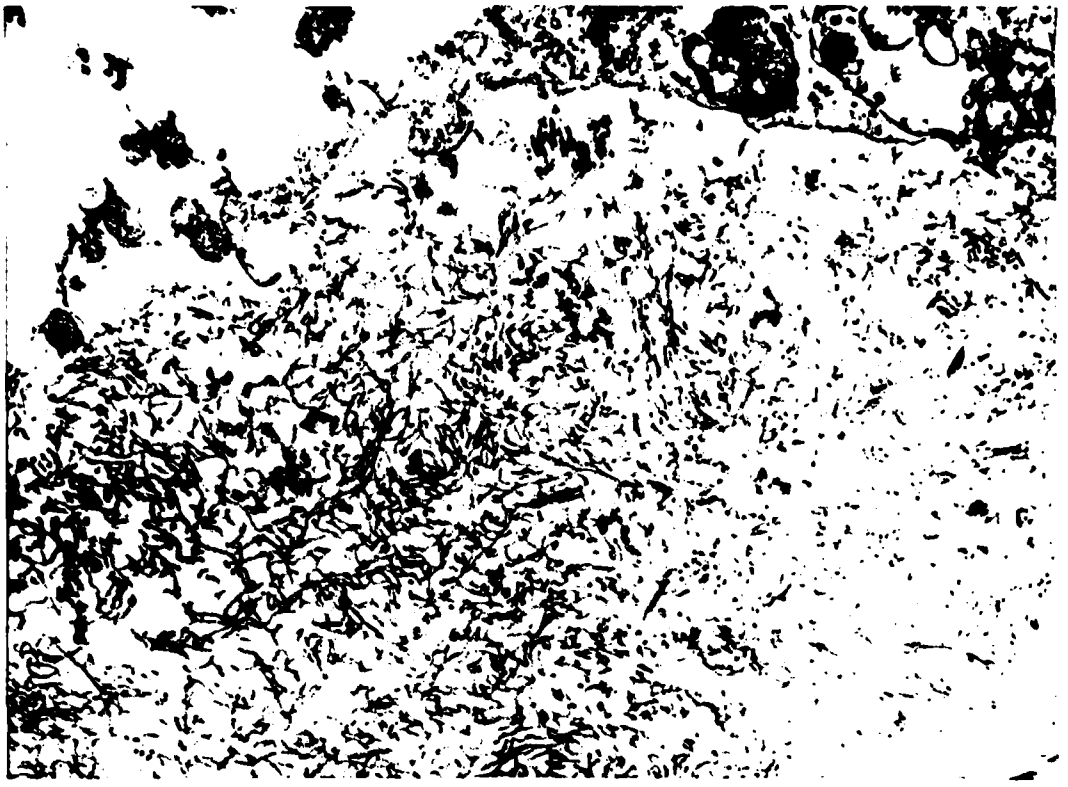


Figure 30. Initial sites of cell fusion were formed after modified areas of plasmalemma (arrows), often with subjacent nucleocapsids (NC) evaginated and contacted an adjacent BFS cell. 84 hours after infection with 72-P-535 leukocyte isolate; 50,000 X.

of plasmalemma or at sites where fusion had been initiated (Figure 31).

Once adjacent cells came into contact and fusion was initiated, small cytoplasmic bridges were formed which progressively enlarged until fusion of adjacent plasmalemmae was completed. Extracellular particles often seen between adjacent cells were never observed acting as an intracellular bridge or as otherwise involved in the fusion process. Although increased numbers of lysosomes were observed within infected cells, a disproportionate increase in lysosomal structures was not observed near areas of membrane fusion.

Cytopathologic Changes of Host Cell Organelles. Due to the cell-associated nature of 72-P-535, it was not possible to examine the sequential development of cytopathic changes induced by viral multiplication in synchronously infected cells. Rather, the following description represents an approximate sequence of the observed ultrastructural changes.

During the first 72 hours after infection, the most frequently observed cellular change was a mild dilatation of the rough endoplasmic reticulum (RER) and the increased production of autophagic vacuoles. Lysosomes increased in number while the Golgi complex and smooth endoplasmic reticulum were not visibly altered. Other organelles and nuclear structures retained the morphological features of those in uninfected cells. Later in the course of infection, lysosomes, autophagic vacuoles and autophagosomes increased in

Figure 31. Cytoplasmic bridges were formed where plasmalemmae of adjacent BFS cells came into contact. Note parallel arrangement of some nucleocapsids (arrow) while others (NC) were arranged randomly. 84 hours after infection with 72-P-535 leukocyte isolate; 50,000 X.

number. Complex proliferations of the RER were the most noticeable reaction to the presence of viral structures during this period. Cytosegrosomes produced by this process often surrounded large collections of nucleocapsids as well as ribosomes and mitochondria (Figures 32,33).

As infection continued, the RER and Golgi complex dilated within a swollen, hydropic cytoplasm. Polysomes were lost from the RER although single ribosomes remained attached. Eventually the SER ballooned drastically which led to an intense vacuolation of the cytoplasm (Figure 34). Residual bodies, multivesicular bodies and myelin figures were produced as areas of focal cytoplasmic degradation predominated (Figure 35). Mitochondria remained relatively unaltered until, in degenerating or necrotic cells, high amplitude swelling and matrical densities were observed.

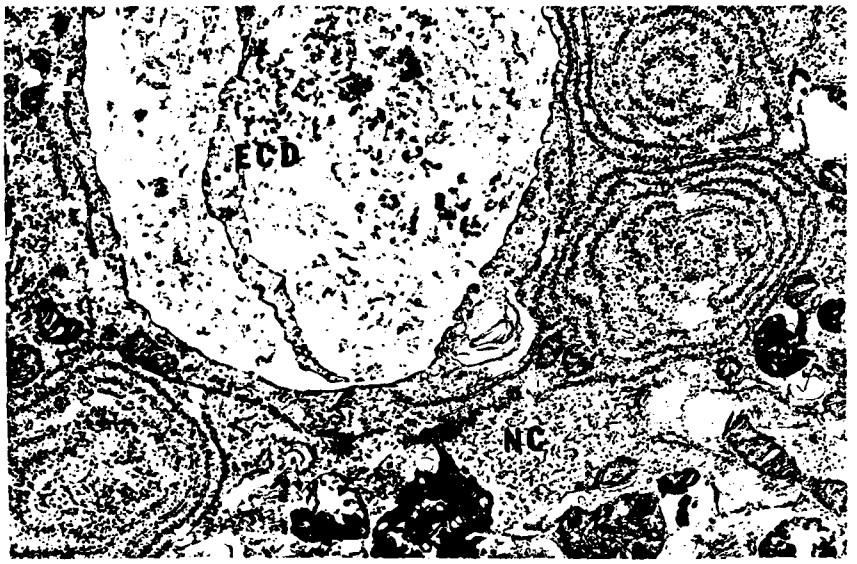
The nuclear membrane remained intact throughout the period of investigation. Some nuclei became pyknotic but, with the exception of the nuclear bodies already described, virus-induced ultrastructural changes were not observed. Nucleoli did not segregate or fragment. Chromatin, which was generally dispersed, marginated only in necrotic cells.

Discussion

In part II, the cytological and antigenic characteristics of a cell-associated virus isolated from cattle with malignant catarrhal fever were described. The virus, 72-P-535, was tentatively identified as a member of the

Figure 32. Concentric whorls of the RER (cytosegrosomes) detected within 72-P-535-infected cells represented a response to cellular injury. (NC) = nucleocapsids; (ECD) = extracellular debris. BFS cell 84 hours after infection; 10,000 X.

Figure 33. Cytosegrosomes sometimes sequestered large accumulations of fuzzy nucleocapsids, ribosomes and mitochondria. 72-P-535-infected BFS cell 84 hours after infection; 30,000 X.


ECD
NC

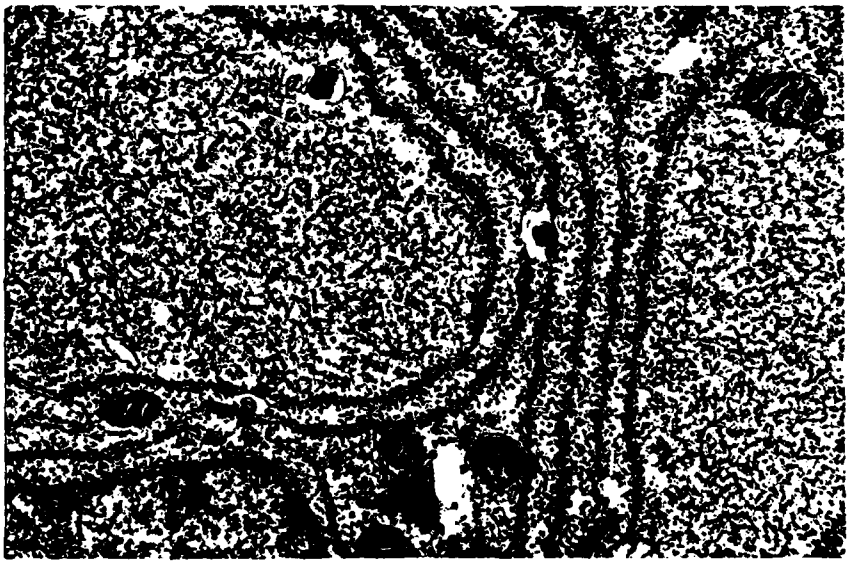
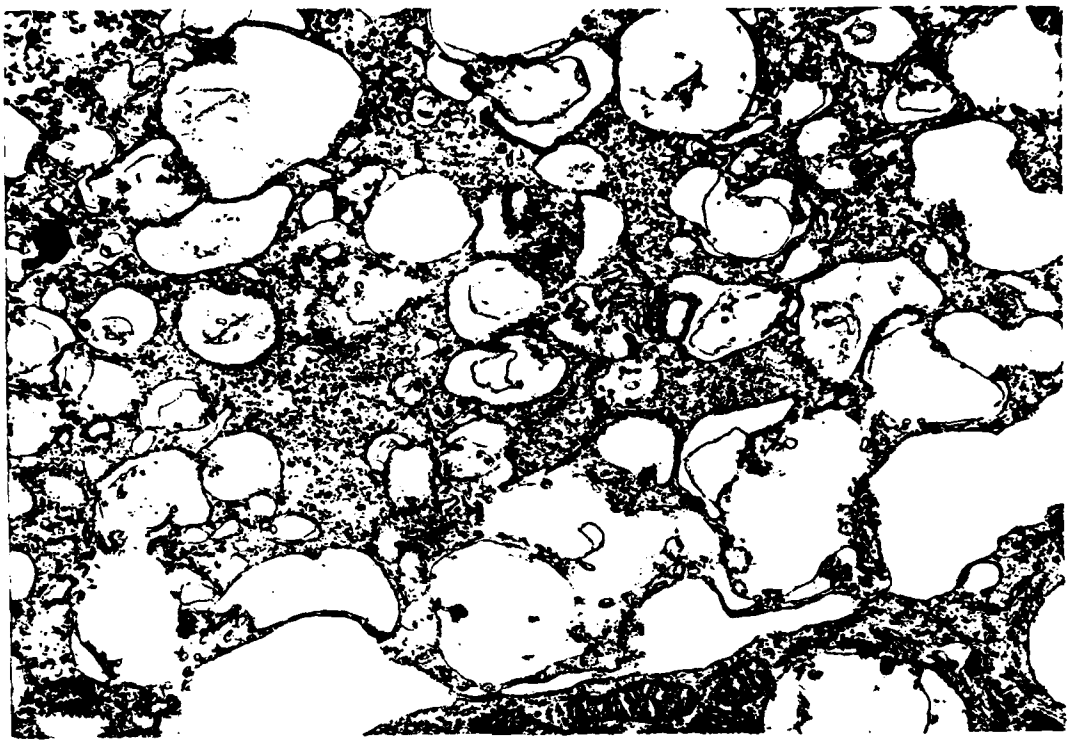
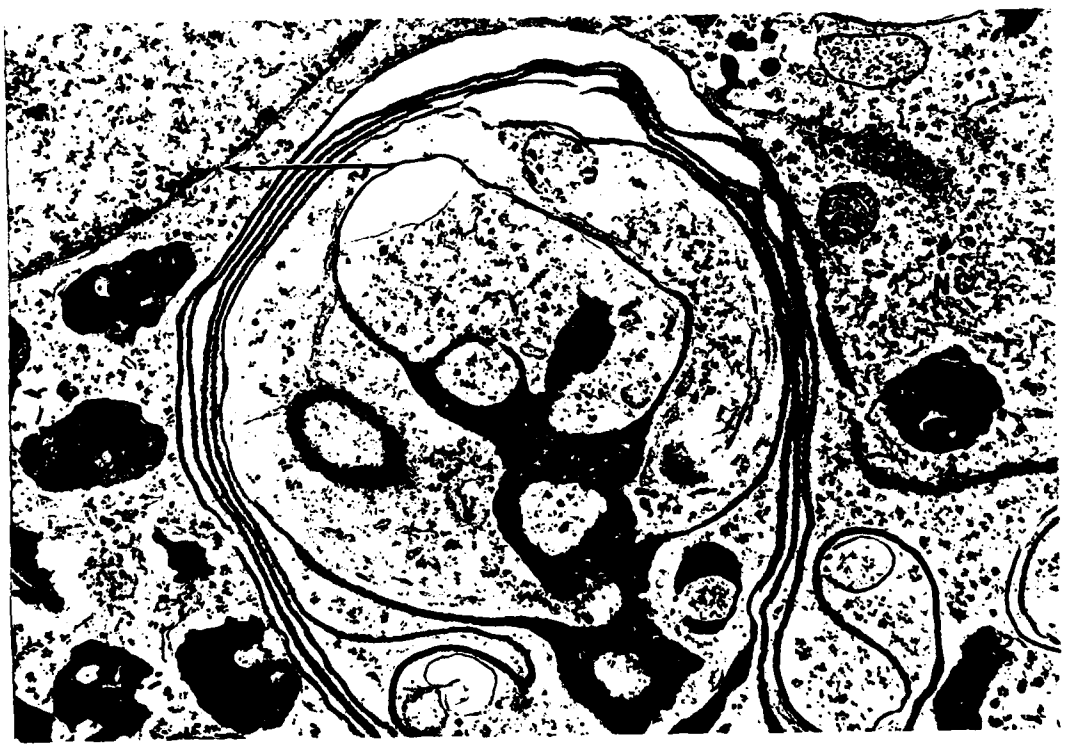


Figure 34. Late in infection the SER ballooned drastically causing an intense vacuolation of the cytoplasm. BFS cell 106 hours after infection; 21,500 X.

Figure 35. Large multilamellar bodies and increased numbers of lysosomes (L) detected in 72-P-535-infected BFS cells indicated that focal cytoplasmic degradation was occurring. (NC) = nucleocapsids; arrows = nuclear membrane. 96 hours after infection; 20,000 X.





morbillivirus family which contains measles, canine-distemper and rinderpest viruses. The ultrastructural characteristics of viral morphology, morphogenesis, cell fusion and other cytopathic changes reported here confirm and extend the previous conclusions.

Sequential development and accumulation of intracytoplasmic nucleocapsids in association with fibrillar material similar to other morbilliviruses (Nakai et al., 1966); Raine et al., 1969; Koestner and Long, 1970; Cornwell et al., 1971; Oyanagi et al., 1971; Tajima et al., 1971b) was found in cells infected with this virus. Intracytoplasmic collections of smooth nucleocapsids have been interpreted to be a consequence of incomplete nucleocapsid morphogenesis in infections which are either non-productive or produce non-infectious virus (Doi et al., 1974; Dubois-Dalcq et al., 1974; Confer et al., 1975b; Lampert et al., 1976; Kratzsch et al., 1977). The consistent appearance of smooth nucleocapsids in degenerating cells may indicate that they are formed after the structural degradation or incomplete synthesis of fuzzy forms.

Virus was released from cells when nucleocapsids and cellular components were enveloped and budded in an exotropic process typical for all members of the paramyxovirus family (Compans et al., 1966; Darlington et al., 1970; Donnelly and Yunis, 1971). Extracellular particles were round or ovoid, contained loosely wound fuzzy nucleocapsids and a continuous 9-11 nm external fringe. Late in infection, particles released from infected cells varied widely in structure and

internal organization. Empty particles were produced as well as those which contained fuzzy or smooth nucleocapsids or a dense granular material. Loosely alveolar particles with a discontinuous external fringe appeared to contain sub-genomic or multi-genomic amounts of nucleocapsids. Similar particles described for morbilliviruses and paramyxoviruses were considered morphologically or otherwise defective (Darlington et al., 1970; ter Meulen et al., 1973; Confer et al., 1975; Lampert et al., 1976; MacIntyre and Armstrong, 1976; Kratzsch et al., 1977).

Intranuclear accumulations of smooth nucleocapsids have frequently been reported in morbillivirus-infected cells (Tajima et al., 1971a, 1971b; Confer et al., 1975b; Lampert et al., 1976) either with or without microfilamentous material forming "simple nuclear bodies" (Llanes-Rodas and Chien, 1965; Nakai and Imagawa, 1969; Dubois-Dalcq et al., 1974; Martinez et al., 1974). Brown and Thormar (1976) presented evidence supporting the earlier hypothesis (Nakai and Imagawa, 1969) that the filamentous material was the direct precursor of smooth nucleocapsids. Such a relationship was not observed during this investigation since microfilamentous material was observed within the nuclei of infected and uninfected cells.

Bratt and Gallaher (1970) identified and defined two basic types of cell fusion; "fusion from within" (FFWI) and "fusion from without" (FFWO) (Poste, 1970, 1972; Knutton, 1978). The two processes are compared in part I of this

dissertation. Cell fusion induced by 72-P-535 was of the FFWI type since released virus was not syncytiogenic at the concentrations achieved and fusion occurred only after intracellular viral products, visualized as thickened plasmalemma, were synthesized. Virus-specific alterations of the plasmalemma promoted the direct fusion of juxtaposed cells after the initial formation of a cytoplasmic bridge. The observed mechanism of polykaryon formation is consistent with the proposed cell-cell bridge model (Hosaka and Shimizu, 1974, 1976).

The ultrastructural changes detected in infected cells indicate that the replication of 72-P-535 does not significantly alter the mormal morphology and presumably the function of host cell components. Hydropic swelling and dilatation of the endoplasmic reticulum are common responses to sub-lethal cellular injury (Cheville, 1976). A variety of mildly injurious chemical or biological agents produce similar initial changes which may or may not lead to cellular necrosis and death (Trump and Arstila, 1975). As described by Hruban and colleagues (1963), the increase in lysosomal structures, autophagosomes, myelin and residual bodies imply that cytoplasmic alterations had occurred leading to an increased turnover of cytoplasmic elements (focal cytoplasmic degradation). The intracytoplasmic sequestration of nucelocapsids and cellular organelles by whorls of endoplasmic reticulum (cytosegrosomes) was further evidence of this process.

Late in infection, after the formation of polykaryons, the intense cytoplasmic vacuolation, mitochondrial changes and dispersion of polysomes indicated that energy-dependent processes supporting macromolecular synthesis and water/electrolyte balance were impaired. The homogeneous distribution of chromatin and interchromatin granules and the structural integrity of nucleoli suggest that nucleus-dependent functions persisted in 72-P-535-infected BFS cells at least until polykaryons degenerated and underwent necrosis.

The specialized procedures required for its isolation and the observed morphologic and biologic properties may indicate that in vivo 72-P-535 existed in a defective, persistent or latent condition and spread by cell-cell contact. It is possible that isolation procedures selected for a single biotype and the in vivo properties of 72-P-535 differ from those observed in vitro. Therefore, investigations into the physical state of the virus in vivo and the mechanism by which it persists and spreads are important areas for further investigation.

Since nucleocapsids produced in infected cells are infectious and able to transmit the infection of adjacent cells, variations in the quantity or quality of nucleocapsids, the composition or integrity of the external envelope fringe may contribute to the lack of infectivity of released particles. Additional antigenic, biochemical and biophysical investigations are required to more accurately identify the defective properties of the virus. Host cell-induced

modifications, temperature sensitivity, thermal lability and the production of defective interfering particles are possibilities which should be examined. Long term animal inoculation studies and the development of a laboratory animal model are needed in order to further evaluate the role of 72-P-535 with respect to malignant catarrhal fever in North America.

Part IV

Isolation and Identification of Viruses Recovered During the MCF California Transmission Study

Introduction

Although MCF has been reported in several midwestern and eastern states, the epizootics reported from Colorado (Pierson et al., 1973a), California and Arizona (Mare, 1977; Orsborn et al., 1978) were particularly severe. Results of virological studies performed after the Colorado epizootic have been previously reported (Storz et al., 1976; Coulter and Storz, 1979). During 1976/1977 three outbreaks of MCF occurred in California dairy herds (Orsborn et al., 1978). The first outbreak occurred in a 1000-cow herd which has been in close contact with sheep. No attempt was made to transmit the agent to calves, sheep or rabbits and virus isolation was not attempted.

Another outbreak involved dairy cattle maintained in close proximity to a zoological garden which housed several species of African ungulates. Blood from affected animals was infectious and produced MCF in susceptible cattle. Subsequently, fourteen consecutive calf-calf transmissions were performed (Mare, 1977). Buffy coat cells obtained from clinically ill animals produced a disease in rabbits which was pathologically consistent with a diagnosis of MCF. Rabbit-derived material also induced MCF in cattle. The transmission and epizootiological data suggest that this outbreak may be attributable to the wildebeest-associated agent.

The outbreak of interest in this investigation occurred several miles from the first mentioned epizootic. Cattle were penned immediately adjacent to a farm which housed ewes

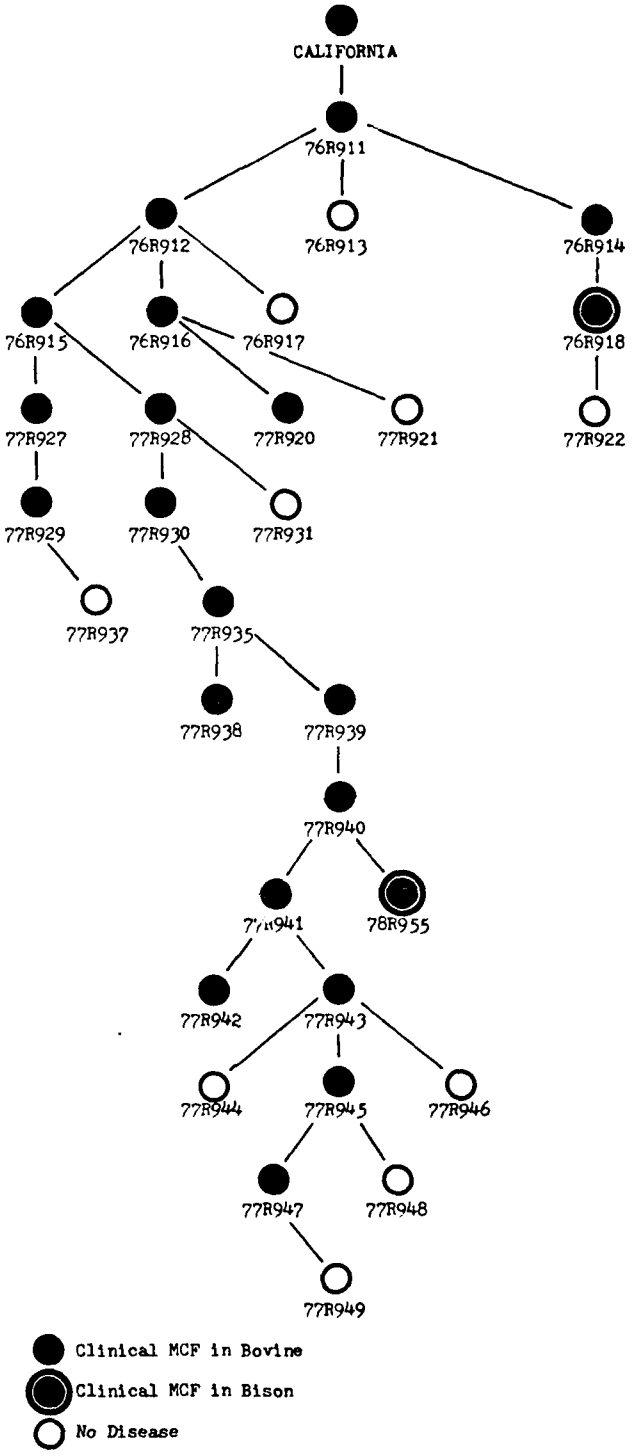
and new lambs. Citrated whole blood was removed from an affected Holstein-Friesian cow and airlifted to Colorado. Upon arrival, some thirty hours later, 500 ml of blood was inoculated into a 160 Kg male Holstein-Friesian calf (76-R-911). Thirty one days later, when the calf had shown signs of illness for three days, blood was withdrawn and used to inoculate three other calves (76-R-912, 76-R-913 and 76-R-914). Beginning with the original inoculum, MCF was eventually transmitted through 12 serial passages to 28 calves and 2 bison using whole blood or the gradient-separated mononuclear cell fraction (Figure 36). Results of these transmission and other pathological studies have been reported by Pierson and coworkers (1978) and by Liggitt and colleagues (1978b, 1979a, 1979b, 1979c).

The purposes of this section are: (1) to summarize the results of virus isolation experiments performed on fluids and tissue samples obtained from these experimental animals and (2) to report the tentative identification of some of the isolates as suggested by electron microscopic and indirect fluorescence analysis of infected bovine fetal spleen cells.

Materials and Methods

Procedures Employed for Isolation of Cell-Associated Virus. At necropsy, following the natural death or euthanization of each animal, selected lymph nodes and samples of various organs, tissues and body fluid were removed

Figure 36. Experimental bovine MCF transmission pattern (from Liggitt, 1978a).


CALIFORNIA
76R911
76R912
76R913
76R914
76R915
76R916
76R917
76R918
77R927
77R928
77R920
77R921
77R922
77R929
77R930
77R931
77R937
77R935
77R938
77R939
77R940
77R941
78R955
77R942
77R943
77R944
77R945
77R946
77R947
77R948
77R949
Clinical MCF in Bovine
Clinical MCF in Bison
No Disease

aseptically, packed in ice and transported to the virus laboratory.

Lymph Node Samples. Lymph nodes were first separated from associated capsular or fatty material and finely minced. Portions were removed and gently ground in a Ten Broeck grinder containing Dulbecco's saline, pH 7.4. When individual cells had been released, cells were collected by centrifugation at 1500 RPM for 10 minutes. Pellets were diluted to 400-500X their original volume and either used to inoculate low passage BFS and BRThyr monolayers, co-cultured with the same cell types or frozen for future use. Co-cultivation included combining infected and uninfected cell suspensions then allowing cells to settle and form a monolayer. Growth medium consisted of MEM supplemented with streptomycin (500 µg/ml), penicillin (500 units/ml) and 10% fetal calf serum (Kansas City Biologicals, Lenexa, KS) or 10% lamb serum obtained from a closed flock maintained at the CSU Animal Experiment Station. Portions of the cell pellet which were to be frozen were diluted to twice their volume in MEM containing 7% DMS and 15% lamb serum then stored under liquid nitrogen in sealed glass ampouls.

Samples of Other Tissues. Samples obtained from other glands or tissues were also separated from associated tissue and minced well prior to processing. After two rapid washings in Dulbecco's saline, minced tissues were placed in trypsinizing flasks containing a magnetic stir bar and

approximately 75 ml of 0.25% trypsin in Ca^{2+}-free Dulbecco's saline. Fluids and cells released during the first 30 minutes of trypsinization were poured-off and discarded. Cells obtained after subsequent 30-45 minute trypsinizations were filtered through sterile gauze and collected by centrifugation at 1500 RPM for 10 minutes. Depending upon its volume, a portion of the cell pellet was diluted to between 10 and 100X its original volume with culture fluid. The resulting suspension of infected cells was either co-cultivated with a suspension of uninfected BFS or BRThyr cells or used to inoculate established monolayers of the same.

Another portion of the original cell pellet was resuspended in enough culture fluid that approximately 0.25 ml packed cell volume could be directly cultivated in plastic tissue culture flasks. Culture medium employed in these direct cultivation procedures consisted of MEM supplemented with 10% heat-inactivated lamb serum from the same closed flock as before. Remaining portions of the cell pellet were either frozen as previously described or discarded.

Blood, Cerebrospinal and Synovial Fluids. At necropsy, blood was collected from animals by cardiac or arterial puncture and stored in glass screw-cap tubes or tubes containing either acid-citrate-dextrose solution or EDTA. Blood collected without anticoagulant was stored at 4 C overnight or until the serum had been expressed from the

clot. Serum was collected, centrifuged at low speen and stored at -20 C.

Citrated or EDTA-treated blood was centrifuged at 2000 RPM for 15 minutes and buffy coat cells were collected. After being washed in Dulbecco's saline and repelleted at low speed, some cells were removed, smeared onto glass slides and stained by the Giemsa method. Aliquots of the remaining cells were used for co-cultivation and monolayer inoculation or frozen under liquid nitrogen as previously described. Cerebrospinal and synovial fluids removed from animals were collected in EDTA tubes. Cells were collected by low speed centrifugation, washed in Dulbecco's saline and repelleted. Smears and inocula were prepared as described.

Infected cell cultures were prepared in duplicate and monitored daily. Observations were recorded. At 7-10 day intervals, monolayers without observable CPE were dispersed with trypsin-EDTA and subpassaged. Approximately 2/3 of the dispersed cells received fresh culture media and were allowed to reform a monolayer. The remaining 1/3 of the cells were used to inoculate established monolayers of uninfected BFS or BFThyr cells. Monolayers which underwent crises during the period of observation were supplanted with uninfected cells as required.

These procedures were carried out through a minimum of 5 tissue culture passages or approximately 8 weeks, after

clot. Serum was collected, centrifuged at low speen and stored at -20 C.

Citrated or EDTA-treated blood was centrifuged at 2000 RPM for 15 minutes and buffy coat cells were collected. After being washed in Dulbecco's saline and repelleted at low speed, some cells were removed, smeared onto glass slides and stained by the Giemsa method. Aliquots of the remaining cells were used for co-cultivation and monolayer inoculation or frozen under liquid nitrogen as previously described. Cerebrospinal and synovial fluids removed from animals were collected in EDTA tubes. Cells were collected by low speed centrifugation, washed in Dulbecco's saline and repelleted. Smears and inocula were prepared as described.

Infected cell cultures were prepared in duplicate and monitored daily. Observations were recorded. At 7-10 day intervals, monolayers without observable CPE were dispersed with trypsin-EDTA and subpassaged. Approximately 2/3 of the dispersed cells received fresh culture media and were allowed to reform a monolayer. The remaining 1/3 of the cells were used to inoculate established monolayers of uninfected BFS or BFThyr cells. Monolayers which underwent crises during the period of observation were supplanted with uninfected cells as required.

These procedures were carried out through a minimum of 5 tissue culture passages or approximately 8 weeks, after

which time isolation attempts were terminated if CPE had not been detected.

When polykaryons were detected in infected monolayers, culture fluids were decanted and the monolayer dispersed with trypsin-EDTA. Cells and polykaryons were collected by centrifugation at 1750 RPM for 10 minutes. The infected culture fluid was clarified by the same procedure. The cell pellet was resuspended in a small volume of MEM containing 2% inactivated lamb serum and added to uninfected BFS monolayers. When numerous polykaryons were present, the process was repeated. On the second or third serial passage after initial isolation, some infected monolayers were dispersed and collected as described then frozen under liquid nitrogen in MEM containing 7% DMSO and 15% inactivated lamb serum.

Monolayers similarly inoculated with clarified culture fluids were observed daily for approximately one week. If polykaryons or other CPE was not detected, cultures were discarded.

Cytological Preparations. Infected monolayers were dispersed, collected by centrifugation, resuspended in culture medium and added to Petri dishes which contained BFS monolayers grown on or without glass coverslips. When numerous polykaryons were present, coverslips were periodically removed and either fixed in Bouin's fluid and stained by the Giemsa method or prepared for immunofluorescence tests (Part II).

Only the 76-R-911 (leukocyte) and 76-R-913 (preinoculation, leukocyte) isolates were grown without glass coverslips and processed for electron microscopic studies described later.

Immunofluorescence Procedures. Indirect immunofluorescence tests were performed in each case. The sources of cow 28, BSV, PI_3 and the rabbit FITC-labeled bovine IgG were identified in the acknowledgements section of this dissertation. The 75-P-2756 serum was obtained from a bison with naturally-occurring MCF. Other sera were obtained from cases of MCF experimentally induced during the California MCF transmission study. Coverslips preparations of infected BFS cells were fixed, reacted with sera and observed as described in part II.

Preparation of Infected Monolayers for Electron Microscopy. Infected monolayers grown without glass coverslips were infected with the 76-R-911 (leukocyte) and the 76-R-913 (pre-inoculation, leukocyte) isolate as described. Approximately 82 hours after inoculation, numerous polykaryons were observed. Infected monolayers were rinsed twice in Dulbecco's saline, fixed in cold cacodylate-buffered glutaraldehyde and processed for electron microscopy as outline in part III.

Results

Virus Isolations. Syncytiogenic viruses were recovered from the lymph nodes, cerebrospinal and synovial fluids,

lymphid and parenchymatous organs of affected cattle (Table 5). Ms. Deryl Keney isolated all viruses except 77-R-941.

Co-cultivation techniques were more reliable than direct cultivation in isolating viruses. Of the 52 isolates obtained during this study, only two (77-R-945 spleen and thyroid) were recovered by the direct cultivation of cells. Of the 50 isolates recovered by co-cultivation, 44 were isolated using BFS cells and only 6 were recovered using BFThyr monolayers.

Viruses were more consistently isolated from leukocytes (62% of samples taken) than from any other source. Therefore, when time or space limitations precluded the examination of large numbers of samples, buffy coat cells were examined exclusively (76-R-916 through 76-R-927). Syncytiogenic, cell-associated viruses were isolated from pre-inoculation and post-inoculation leukocytes of experimental MCF calf 76-R-913. All California MCF isolates were strictly cell-associated. Infectivity was not detected within cell-free culture fluids.

Cytological Characteristics. Each of the 52 California MCF isolates produced polykaryons in low passages BFS monolayers. With on exception, (77-R-941), polykaryons produced by California MCF isolates differed from the 72-P-535 type both in the number and arrangement of nuclei. The 76-R-911 isolate briefly described in part II is representative. Polykaryons of the 76-R-911 type rarely contained more than 30 nuclei unless secondary fusion had occurred. Nuclei were

Table 5. Polykaryon-Forming Viruses Recovered from Cattle with Experimental MCF (MCF California)

Samples Tested	76-R-911		76-R-912		(No MCF) 76-R-913		76-R-914		76-R-915		76-R-916		76-R-917		Bison 76-R-918		76-R-927	
	BFS	BFThyr	BFS	BFThyr	BFS	BFThyr	BFS	BFThyr	BFS	BFThyr	BFS	BFThyr	BFS	BFThyr	BFS	BFThyr	BFS	BFThyr
Leukocytes	+(5/6)	-	+(3)	-	+(5*)	-	-	-	+(5)	-	+(5)	+(4)	+(4)	+(4)	+(3)	NT	+(6)	NT
Adrenal	+(5)	-	NT	NT	-	+(3)	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT
Spleen	+(4)	-	NT	NT	+(4/5)	-	+(5)	-	+(5)	-	NT	NT	NT	NT	NT	NT	NT	NT
Thyroid	NT	NT	NT	NT	-	-	+(6)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Kidney	+(5)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Ependyma	+(5)	-	-	-	+(4)	+(3)	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT
Synovial Fluids:																		
Unspecified	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	+(6)	-
Left Carpal	NT	NT	+(5)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Right Carpal	NT	NT	+(5)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Right Shoulder	NT	NT	+(5)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Hock	NT	NT	NT	NT	+(5)	+(3)	+(5)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Right Elbow	NT	NT	NT	NT	+(3)	+(3)	+(3)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Right Tarsal	NT	NT	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
CSF	NT	NT	-	-	-	-	+(4)	-	-	-	-	-	-	-	-	-	+(6)	-
Peritoneal Fluid	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-
Lymph Nodes:																		
Longissimus	+(4)	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Subilicus	+(5)	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	NT	NT	NT	NT	NT	NT
Mesenteric	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+(6)	-	NT	NT	NT	NT	NT	NT
Bone Marrow	NT	NT	NT	NT	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Trigeminal Ganglion	NT	NT	NT	NT	NT	NT	NT	NT	-	-	NT	NT	NT	NT	NT	NT	-	-

*Syncytiogenic virus recovered from pre-inoculation leukocytes.

- indicates no virus recovered

+(5) indicates polykaryons detected at 5th subpassage

NT indicates not tested

BFS = bovine fetal spleen cells

BFThyr = bovine fetal thyroid cells

Table 5. (Continued)

Samples Tested	(Pre-Inoculation) 77-R-93? BFS	77-R-93? BFThyr	76-R-934 BFS	76-R-934 BFThyr	77-R-935 BFS	77-R-935 BFThyr	77-R-938 BFS	77-R-938 BFThyr	77-R-939 BFS	77-R-939 BFThyr	77-R-941 BFS	77-R-941 BFThyr	77-R-943 BFS	77-R-943 BFThyr	77-R-945 BFS	77-R-945 BFThyr	77-R-947 BFS	77-R-947 BFThyr
Leukocytes	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Adrenal	-	-	NT	NT	NT	NT	NT	NT	NT	NT	+(5)	NT	-	-	-	-	-	-
Spleen	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	Direct (2)		-	-
Thyroid	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+(8)	-	Direct (2)		+(2)	-
Kidney	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	NT	NT	NT	NT
Synovial Fluids:																		
Unspecified	+(5),-(6)	NT	NT	NT	NT	NT	NT	NT	+(5)	-	NT	NT	+(6)	-	-	-	NT	NT
Hock	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+(2)	-
Lymph Nodes:																		
Longissimus	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	NT	NT	NT	NT	+(4)	-
Mesenteric	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+(4),-(5)	-
Prefemoral	NT	NT	NT	NT	NT	NT	NT	NT	+(5)	NT	NT	NT	NT	NT	NT	NT	-	-
Hepatic	NT	NT	NT	NT	NT	NT	NT	NT	+(5)	NT	NT	NT	NT	NT	NT	NT	NT	NT
CSF	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	+(6)	-	-	-	+(3)	-
Peritoneal Fluid	-	-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+(6)	-	-	-	NT	NT
Lymph Fluid	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	NT	NT	NT	NT	NT	NT
Lymphocytes	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	+(6)	-	+(2)	-
Urine Sediment Cells	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	NT	NT	NT	NT	NT	NT

Note: Uninoculated BFS and BFThyr monolayers were negative in every case.

- indicates no virus recovered

+(5) indicates polykaryons detected at 5th subpassage; +(5),-(6) indicates that polykaryons were detected at the 5th subpassage but not at the 6th

NT indicates not tested

BFS = bovine fetal spleen cells

BFThyr = bovine fetal thyroid cells

usually arranged peripherally within polykaryons rather than in a centrally located and random manner (Figure 4). As with 72-P-535, polykaryons were first observed 24-36 hours after subpassage. Polykaryons grew up by the successive recruitment (fusion) of peripheral mononucleated cells. Approximately 72 hours after inoculation, the cytoplasm became vacuolated and began to retract. Later, the polykaryon detached from the surface of the culture vessel. Neither nuclear nor cytoplasmic inclusions were ever observed in Giemsa stained preparations.

Results of Immunofluorescence Analysis.

Controls. Specific fluorescence was observed when antiserums to PI_3 (70-P-1096), a bovid morbillivirus (72-P-535) and a herpesvirus (75-R-2756) were reacted with homologous antigens. Specific fluorescence was not observed using test serums and uninoculated BFS cells.

Test Samples. Using BSV antiserum and the described indirect fluorescence procedure, similar fluorescence patterns were observed with each of the California MCF isolates tested. Since chronological time points were not taken, no attempt was made to document the sequential distribution of antigens in infected cells.

Results of immunofluorescence analysis of infected BFS cells summarized in Table 6 indicate that most California MCF isolates represent members of the ubiquitous bovine syncytial virus group. Isolates recovered from MCF cattle subsequent to 76-R-913 which induced polykaryons of the same

Table 6. Results of Indirect Immunofluorescence Tests Using Viruses Isolated from Experimental (MCF California) Cattle and Antiserums to Selected Polykaryon-Forming Viruses

ANTIGENS	ANTISERUMS							
	Cow 28	PI_3	75-R-2756	76-R-911	(Pre-Inoc) 76-R-913	(Post-Inoc) 76-R-913	BSV	Control
VIRUS CONTROLS								
72-P-535 (Morbilli)	<32	<8*	<8*	<8*	16	8	≤16	NEG
70-P-1096 (PI_3)	< 4	16	<8*	NT	NT	NT	< 8*	NEG
75-P-2756 (Herpes)	NT	NT	≥32	<8*	<8*	8	>64	NEG
VIRUS ISOLATES								
76-R-911 (leukocyte)	>4	<8*	<8*	16	>32	32	>32	NEG
76-R-912 (leukocyte)	NT	NT	NT	NT	NT	NT	>16	NEG
76-R-913 (leukocyte, pre-inoc)	NT	<8*	<8*	>32	>32	>32	32	NEG
76-R-914 (spleen)	NT	NT	NT	NT	NT	NT	≤ 8	NEG
76-R-915 (leukocyte)	NT	NT	NT	NT	NT	NT	≥16	NEG
76-R-918 (leukocyte)	NT	NT	NT	NT	NT	NT	≥16	NEG
76-R-927 (leukocyte)	NT	NT	NT	NT	NT	NT	≥16	NEG
76-R-938 (synovial fluid)	NT	NT	NT	NT	NT	NT	≥16	NEG
77-R-939 (prefemoral node)	NT	NT	NT	NT	NT	NT	≥16	NEG
77-R-941 (adrenal)	NT	<8*	NT	NT	NT	NT	≥16	NEG
Uninfected BFS Control	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

*=lowest dilution tested

Reported results are the reciprocal of the highest dilution with which specific fluorescence was detected.

morphology and staining properties were first screened against the BSV antiserum. Except for isolate 77-R-941, any virus isolated subsequent to 76-R-913 which reacted specifically with BSV antiserum was tentatively identified as a bovine syncytial virus and no other tests were performed.

Antiserums to PI_3 or 75-R-2756 did not react when applied to BFS cells infected with the representative 76-R-911 isolate. Besides its previously reported titer of antibody to isolate 72-P-535 (Part II), cow 28 serum also reacted with 76-R-911 antigens. The antibody titers to 76-R-911 detected in BSV antiserum and serum from calf 76-R-913 support the electron microscopic identification of these isolates reported later.

In BFS cells infected with 76-R-911, the juxtanuclear cytoplasm was often the brightest area and a diffuse granular fluorescence of the entire cytoplasm was consistently observed in both polykaryons and infected peripheral cells. Brightly fluorescing areas of particulate material or dark areas ringed by a bright halo were distributed throughout the cytoplasm (Figure 7). Nuclei within polykaryons frequently did not react although in small polykaryons fluorescence was often associated with the nuclear membrane or detected as discrete foci within the nucleoplasm. Specific fluorescence of the plasmalemma was not seen.

As reported in part II and elsewhere, polykaryons with two distinct morphologies were detected in the 77-R-941 (adrenal) isolate. Antiserum to BSV reacted with at least

one of the morphological types. At this writing, 77-R-941 has not been reacted with cow 28 antiserum.

Isolate 76-R-913 is of particular interest since it was recovered from both pre-inoculation and post-inoculation leukocytes. Results of immunofluorescence tests of pre-inoculation and post-inoculation sera from the same animal, serum from calf 76-R-911, bison 75-R-2756 and BSV antiserum indicate that a bovine syncytial virus was present in this animal prior to it contracting MCF.

Ultrastructural Features of Virus-Infected BFS Cells. Electron microscopic observations of BFS cells infected with the 76-R-911 and 76-R-913 isolates were made only as a further aid in their identification. A complete study of the morphogenesis and cytopathology of the viruses was not undertaken. Virus particles were produced and released by polykaryons and infected single cells. Nucleocapsids detected in these cells were circular or hexagonal in profile, 40-45 nm in diameter and had an electron-lucent center. They were also detected free in the cytoplasm as well as associated with the endoplasmic reticulum, plasmalemma or other intracytoplasmic membranous structures (Figure 37).

Depending upon the site at which an envelope was acquired, either single or double-membraned enveloped particles were observed. Frequently, both forms were observed within the same cell. Nucleocapsids with a double-membraned envelope were observed budding completely through the tortuously curved rough and smooth endoplasmic reticulae. The

resultant cytoplasmic particle consisted of a central 40-45 nm nucleocapsid surrounded successively by an 11-15 nm translucent area, a membrane, a 25-30 nm area identified as the cistern of the endoplasmic reticulum and an outer limiting membrane (Figure 37). Often, two or more nucleocapsids were surrounded by a common double membrane. When only one nucleocapsid was present, the overall diameter of the particle was between 127 and 150 nm. Nucleocapsids were not detected within the cisternae of the endoplasmic reticulum and particles with this morphology were not observed extracellularly.

Extracellular single-enveloped particles were produced after nucleocapsids aligned beneath modified areas of plasmalemma and budded in a classical manner. Nucleocapsids infrequently acquired envelopes by budding into membrane bound cytoplasmic vesicles. Particles produced by either budding process contained the same 40-45 nm virus core as described for the endoplasmic reticulum-associated (ERA) form. The envelope of the single-membrane enveloped particles contained a hazy 12-15 nm external fringe. The overall diameter of this type of particle was approximately 90 nm (Figure 38). As with the double-membraned form, particles which contained 2-6 nucleocapsids were observed.

In addition to viral nucleocapsids, large rounded and vacuolated structures composed of granular material were detected only in infected cells. Their close proximity to nucleocapsids suggests that these structures may represent

"virus factories" or areas which contain materials from which nucleocapsids are formed (Figure 38).

Discussion

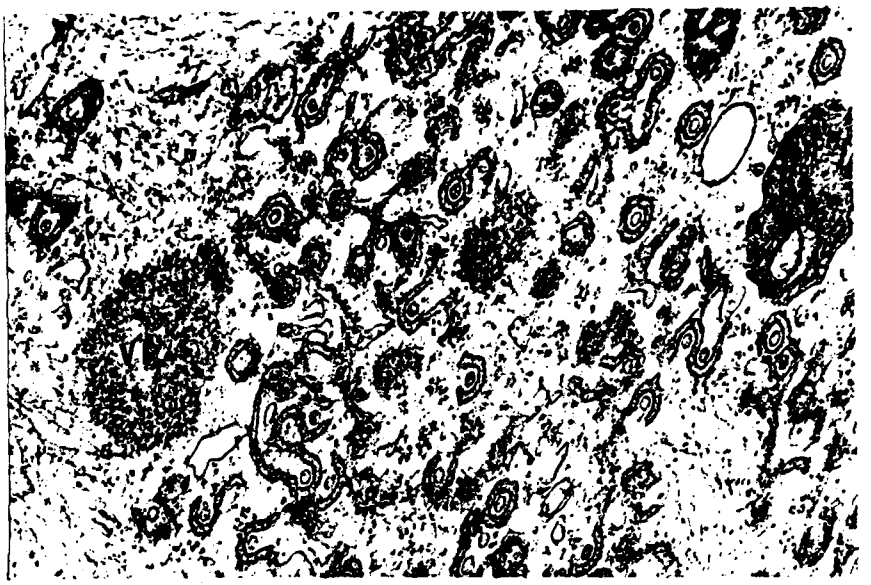
Syncytiogenic, cell-associated viruses were isolated from a wide variety of fluid, tissue and organ samples of cattle investigated during the California MCF transmission study (Liggit, 1978a). Many of the viruses isolated were recovered from tissues of the types successfully employed by Storz and colleagues (1976) during the 1972 Colorado epizootic and by other researchers who have isolated viruses from MCF animals (Plowright, 1968; Clarke et al., 1973). The results of virus isolations reported in this study agree with the findings of Malmquist and colleagues (1969) that the co-cultivation of leukocytes with BFS cells constitutes an efficient process for the isolation of cell-associated syncytiogenic viruses from cattle.

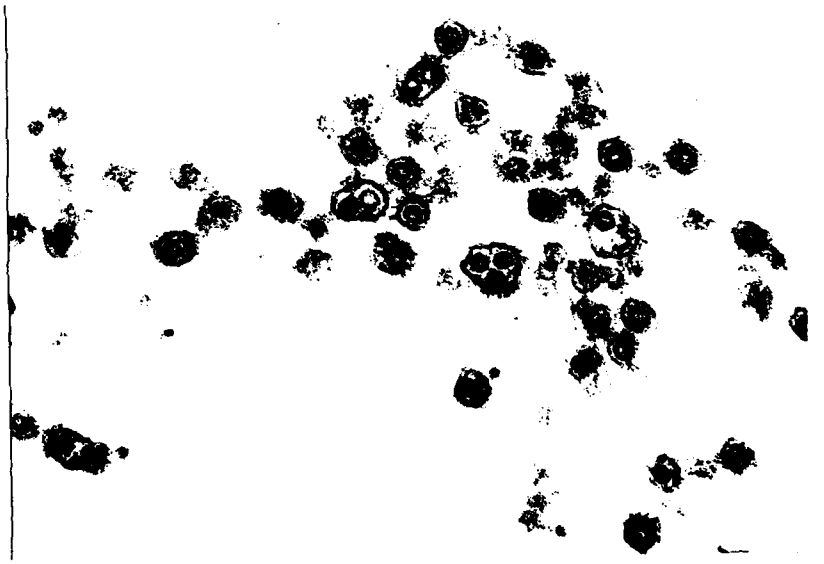
Virus was also recovered from the synovial, cerebrospinal and peritoneal fluids and from gradient-separated lymphocytes after co-cultivation with BFS or BFThyr monolayers. Several investigators have confirmed the efficiency and applicability of co-cultivation procedures in the isolation and propagation of cell-associated viruses (Malmquist et al., 1969; Estes et al., 1969; Woode and Smith, 1973).

The cytological, immunofluorescence and electron microscopic analyses reported in this study indicate that, with the exception of the 77-R-941 isolate, each of the California

Figure 37. Nucleocapsids of 76-R-911 leukocytes isolate budded through the endoplasmic reticulum of infected BFS cells produced a double-membraned particle typical of BSV. Some envelopes contained multiple nucleocapsids. VF = viral factory. 72 hours after infection; 36,000 X.

Figure 38. Single-enveloped particles were released extracellularly from BFS cells infected with California MCF isolate 76-R-911. Multiploid particles and an external fringe were evident. 72 hours after infection; 71,000 X.





MCF isolates is a member of the bovine syncytial virus group (Malmquist et al., 1969; Estes et al., 1969; Boothe et al., 1970). The polykaryons produced by BSV in BFS monolayers had the same morphology and staining properties as the described California isolates (Malmquist et al., 1969; Scott et al., 1973; Gillette and Olson, 1973). As described in this study and previously reported by Woode and Smith (1973), rapidly growing monolayers facilitated the growth of BSV-induced polykaryons while polykaryons induced by 72-P-535 were best propagated in stationary phase monolayers (Part III).

Immunofluorescence patterns observed in infected cells agreed with patterns previously described for BSV (Malmquist et al., 1969; Boothe et al., 1970; Woode, 1972; Smith 1973; Gillette and Olson, 1973). A characteristic nuclear fluorescence was also reported in many of the previously cited papers. Nuclear fluorescence was infrequently observed during this study although a chronological study of the appearance of viral antigens was not made.

In their original report, Malmquist and colleagues (1969) used both direct and co-cultivation techniques to isolate BSV from lymph nodes, buffy coat, milk sediment and splenic samples of lymphosarcomatous and clinically normal cattle. Electron microscopic analysis of infected cells detected single-membraned enveloped particles budding both from the plasmalemma and into cytoplasmic vacuoles. In either case, virus particles were 90-115 nm in diameter

including a fringe of 14-18 nm surface projections. The centrally-located nucleocapsid was described as "essentially spherical" and 35-45 nm in diameter. Enveloped particles containing multiple nucleocapsids were observable in their electron micrographs.

Estes and coworkers (1969) also used co-cultivation techniques to recover BSV from samples of lymphosarcomatous cattle. Double-membraned enveloped virus particles 120 nm in diameter were detected in infected BFS cells. The nucleocapsid was 30-40 nm in diameter. Boothe and associates (1970) observed that both single and double-membraned particles were often present in the same cell and postulated that the endoplasmic reticulum-associated (ERA) particles were an aberrant form of the single-membraned form. The latter were 90-115 nm in diameter with a 14-18 nm external fringe. Double-membraned (ERA) particles were 110-150 nm in diameter and contained nucleocapsids which were indistinguishable from those of the single-membraned form. The same researchers also reported rounded intracytoplasmic granular structures similar to those reported here associated with developing nucleocapsids. Areas corresponding to these structures fluoresced in indirect immunofluorescence tests and were presumed to contain viral precursors.

In Northern Ireland, Clarke and McFerran (1970) observed that the morphology, morphogenesis and antigenic distribution of BSV closely paralleled patterns described for simian foamy viruses (Clarke et al., 1969; Fleming and Clarke, 1970).

Based on their electron microscopic observations, Dermott and coworkers (1971) supported the inclusion of BSV in the group which contains simian foamy viruses and the feline and human syncytial viruses. The same authors reported that, in BHK-21 cells, BSV nucleocapsids were first formed within the nucleus where they were detected in association with chromosomes. No evidence of nuclear involvement was observed when the same virus was propagated in BFS cells. This may not represent a variation in the behavior of BSV in the two cell types since the authors also reported that at least one other type of virus was also present in the BHK-21 cell line. In Northern Ireland, Clarke and associates (1973) isolated BSV from the spleen of an MCF animal. The extracellular particles were 95 nm in diameter with a 12-13 nm external fringe. Several nucleocapsids were observed within the same envelope. These authors supported the inclusion of BSV within the foamy virus group (Parks and Todaro, 1972).

Scott and colleagues (1973) isolated BSV from lung, kidney, uterus and buffy coat samples of adult cows and from the spleen and kidney samples of bovine fetuses. They confirmed the presence of "discrete double membrane-bound viral particles approximately 135 nm in diameter" within the cytoplasm of infected cells.

The results presented in this study indicate that the 76-R-911 and the 76-R-913 isolates are members of the bovine syncytial virus group. Seroepidemiological findings suggest that as many as 25-30% of American cattle may be inapparently

infected (Malmquist et al., 1969; Scott et al., 1973). Antibodies to 76-R-911 were detected in the 72-P-535 reference serum (cow 28) indicating that cow 28 had been previously exposed to BSV.

Bovine syncytial virus has been isolated from clinically normal cattle as well as cattle affected with MCF or lymphosarcoma but has not been proven to cause a disease acting alone. Perhaps the importance of a persistent infection with BSV is not associated with an ability of the virus to directly cause disease but rather with an ability to predispose the animal to diseases from other sources. The presence of a multiple mixed infection involving BSV may alter the immune response of the animal, cause the recrudescence of a latent virus or act synergistically to produce a disease not characteristic of either virus acting alone.

Further characterization of each of the California MCF isolates is required. The isolation of BSV in association with another cell-associated virus of the 72-P-535 type is of particular interest and requires further confirmation and investigation.

Part V

Centrifugation Analysis of 72-P-535-Infected Cells and Culture Fluids

Introduction

Morbilliviruses are frequently cell-associated upon initial isolation (Fraser and Martin, 1978) but may become cell-released when subsequently subpassaged, co-cultivated or fused with susceptible cells (Horta-Barbosa et al., 1970, 1971). With some strains, infectivity remains associated with the cell either because extracellular virus is not released (non-virogenic) or because released virus is not infectious (Burnstein et al., 1974; Doi et al., 1974; Kratzsch et al., 1977).

As reported in part II, the infectivity of isolate 72-P-535 remained strictly cell-associated through 49 serial subpassages. The morphological or morphogenic basis of this cell-association was first investigated by the electron microscopic analysis of thin sectioned infected cell (Part III). In this study, the further electron microscopic analysis of negatively stained material from infected cells and culture fluids confirm earlier findings. Non-infectious particles released into culture fluids are similar in size and morphology to other morbilliviruses. These enveloped particles and the nucleocapsids released from disrupted infected cells represent the only detectable virus-specific products in infected cell cultures.

Materials and Methods

Analysis of Virus-Specific Structures in Infected Cells. Five Blake bottles (Belco Glass, Inc., Vineland, NJ)

containing 72-P-535-infected low passage BFS monolayers were scraped from the glass with a rubber policeman. Infected cell suspensions were partially clarified by centrifugation at 1750 RPM for 15 minutes at room temperature. Supernatant fluids were frozen at -20 C and retained for future analysis. Pellets of infected cells were resuspended in 30 ml of reticulocyte standard buffer (0.01 M Tris-HCl, 0.01 M KCl, 0.0015 M $MgCl_2$; pH 7.4) at 4 C. Suspensions of osmotically-swollen cells were disrupted by 50 strokes of a tight-fitting Dounce homogenizer ("B" plunger). Cell disruption was monitored by phase microscopy. Disrupted cells were then centrifuged at 1000 RPM for 5 minutes to remove nuclei and coarse debris.

The supernatant fluid was added above 5 ml of a 40% (w/w) sucrose cushion and centrifuged at 21,000 RPM for 2.75 hours in an SW-40 rotor (Beckman Instruments, Palo Alto, CA). It was previously calculated that 2.65 hours at this speed would pellet materials 100 S or larger. The resultant pellet was resuspended in 6 ml of TE buffer (0.005 M Tris-HCl, 0.001 M EDTA), pH 7.4. Aliquots (1.5 ml) were layered into 9/16" x 3 3/4" cellulose nitrate centrifuge tubes containing a linear 15-65% (w/w) sucrose gradient. Tubes were placed in a Beckman SW-40 rotor and centrifuged at 30,000 RPM (160,000 g) for 18 hours at 4 C.

Serial drip fractions were collected by pinhole puncture and the density of each fraction was determined by refractive index (Bausch and Lomb, Inc., Houston, TX). After dialysis

against a large excess of 1% ammonium acetate for 12 hours at 4 C, fractions were concentrated to less than one ml by dialysis against polyethylene glycol 30,000 (m.w. 20,000).

Formvar-carbon coated grids were degreased by immersion in carbon tetrachloride. After drying, one drop of each fraction was added to a grid and allowed to remain for two minutes. Excess fluid was drawn off with blotting paper and one drop of freshly filtered 3% aqueous phosphotungstate, pH 7.2, was applied for one minute. Excess stain was removed before grids were dried overnight at room temperature under vacuum (-15 psi). Specimens were observed using an Hitachi HU-12 electron microscope operated at 75 Kv.

Analysis of Cell-Free Culture Fluids. After being stored at -20 C, supernatant fluids collected from Blake bottles of infected BFS monolayers were thawed and examined electron microscopically as described in part II of this dissertation.

Results

Virus-Specific Structures in Infected Cells. A total of 37 serial drip fractions were negatively stained and observed. Fraction densities ranged from 1.01 to 1.32 gm/cm^3. The appearance of a cellulose nitrate tube after centrifugation is depicted in Figure 39a. The bands at 7.0 and 7.7 cm from the bottom of the tube contained large aggregates of cellular debris and, presumably, cellular

Figure 39a. Representation of cellulose nitrate tube used in analysis of virus-specific structures within 72-P-535-infected BFS cells. Tube contained linear 15-65% (w/w) sucrose gradient and was centrifuged at 160,000 g for 18 hours at 4 C. Nucleocapsids and associated membranous material banded at densities of 1.25-1.27 gm/cm^3.

Figure 39b. Representation of cellulose nitrate tube used in analysis of virus-specific structures within cell-free infected culture fluids. Enveloped virus particles were distributed throughout the cloudy central portion of the tube. Linear 20-40% (w/w) sucrose gradient centrifuged at 81,000 g for 2 hours at 4 C.

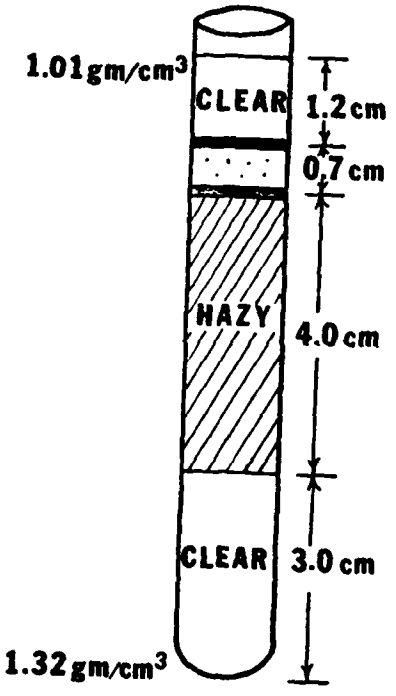


Figure 39a.

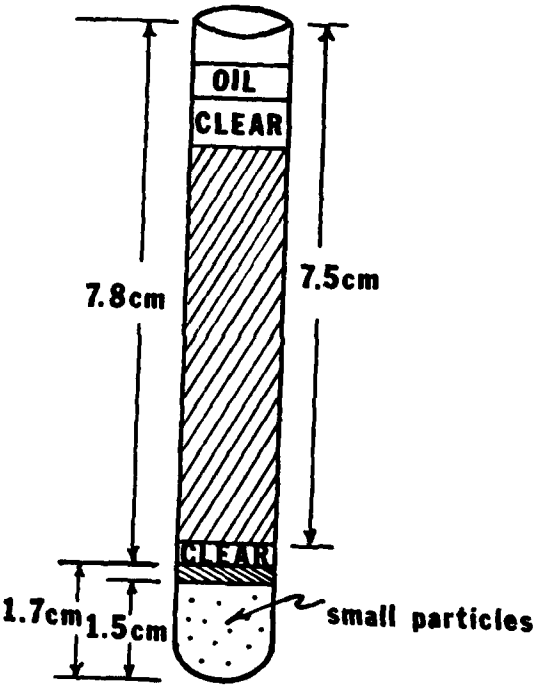


Figure 39b.

organelles. Their large size and electron density made photography impossible.

Virus-specific structures identified as helical nucleocapsids were detected in fractions 7 through 11, corresponding to densities of 1.24 to 1.27 gm/cm^3. Although nucleocapsids occurred as large aggregates and were often associated with membranous material, they were easily identified and measured. As reported in part II, nucleocapsids were 17-20 nm across with distinct 5-7 nm surface striations (Figure 40).

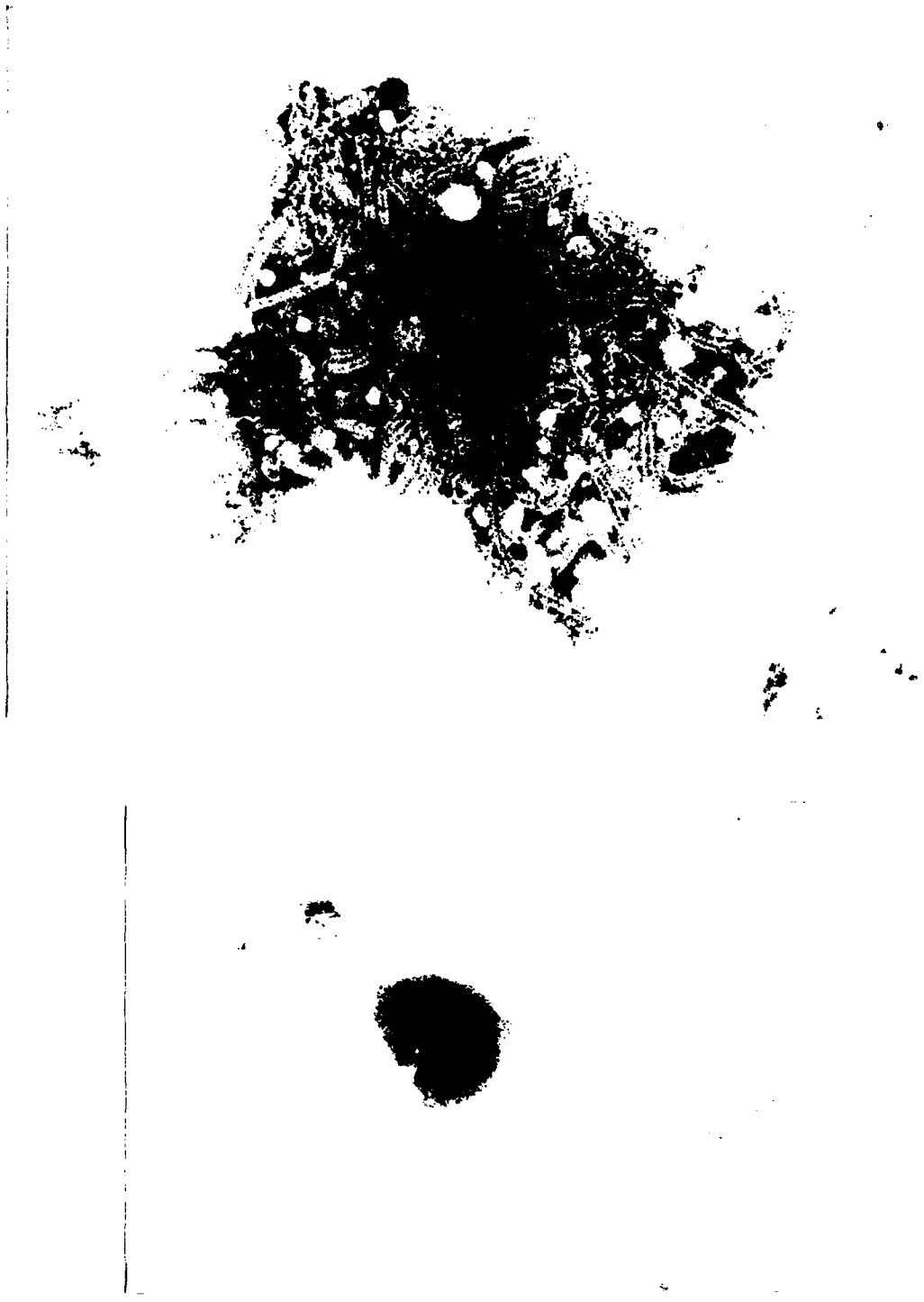
No other virus-specific structures were identifiable although peculiar grape-like clusters of unknown origin were present in the same fractions as nucleocapsids (Figure 41).

Viral Structures Identified in Cell-Free Culture Fluids. The appearance of a representative cellulose nitrate tube after centrifugation is represented in Figure 39b. A total of twelve serial drip fractions were obtained and analyzed.

Rounded or ovoid enveloped virus particles 150-500 nm in diameter were scattered throughout the cloudy portion of the tube. The greatest concentration of particles was detected in fractions 5 and 6, corresponding to the center of this region. Helical tubular structures identified as nucleocapsids were loosely wound inside intact particles or released from particles which had been disrupted. Nucleocapsids were 17-20 nm in diameter with obvious 5-7 nm surface striations (Figures 12,13). Often, the patchy 9-11 nm fringe of surface projections was not detected in intact or disrupted 72-P-535 particles.

Figure 40. Large aggregates of nucleocapsids with a density of 1.24-1.27 gm/cm^3 were the only virus-specific structures released from disrupted 72-P-535 infected BFS monolayers; 189,000 X.

Figure 41. Grape-like clusters of unidentified material were present in the same serial fractions as nucleocapsids (above); 120,000 X.



Discussion

Virus-specific structures detected in 72-P-535-infected cells and culture fluids correspond in size and morphology to extracellular particles and nucleocapsids previously described from thin sectioned material (Part III). Observations of negatively stained preparations confirm earlier findings that 72-P-535 is an enveloped virus containing a helically symmetrical nucleocapsid consistent with its classification within the morbillivirus genus of the family paramyxoviridae.

Without the serological findings presented in part II, these morphological findings alone would not be sufficient to make such a classification. Virus components similar to those described here have also been reported from negatively stained preparations of other genera within the family (Horne and Waterson, 1960; Waterson et al., 1961a, 1961b; Compans and Choppin, 1967; Klenk and Choppin, 1969; Finch and Gibbs, 1970).

Using the negative stain procedures of Brenner and Horne (1960) or Almeida and Howatson (1963), other researchers have described viral components similar to those reported here within or released from a variety of cells infected with measles, canine distemper or rinderpest viruses. Norrby and colleagues (1963) reported that intact CDV virions were highly pleomorphic, 150-300 nm in diameter and contained striated nucleocapsids approximately 18 nm in

diameter. Surface projections which ringed the periphery of the enveloped particles were 11-15 nm in length.

Tajima and colleagues (1971b) compared and morphology of CDV and rinderpest virions in thin-sectioned and negatively-stained material. They confirmed the pleomorphism of CDV virions. Intact particles ranged in size from 160-900 nm. "Herring-bone" patterned nucleocapsids 19 nm in diameter were detected within enveloped particles which had 16 nm projections arranged at the periphery.

Since the pioneering work of Enders and Peebles (1954), the structural and biophysical characteristics of the measles virion have been investigated by numerous researchers (Waterson et al., 1961b; Waterson, 1965; Norrby and Magnusson, 1965; Norrby and Hammarskjold, 1972; Nakai and Imagawa, 1969; Nakai et al., 1969; Hall and Martin, 1973). A summary of the available evidence indicates that measles virions are highly pleomorphic and vary in diameter between 100 and 300 nm. Each virion contains a loosely wound nucleocapsid and an external 16-17 nm fringe (Fraser and Martin, 1978). Thorne and Dermott (1976) observed circular and elongated linear nucleocapsids released from disrupted measles virus-infected cells. Similar structures were not detected during this study nor have they been reported from cells infected with rinderpest virus or CDV.

Although few studies of the structural or biophysical characteristics of rinderpest virus have been reported, available results indicate that the components of the

rinderpest virus are nearly identical to those reported for 72-P-535, CDV and measles virus (Underwood and Brown, 1974). Plowright and associates (1962) investigated the ultrastructural characteristics of the rinderpest virion grown in bovine fetal kidney monolayers. They reported that virions of the Kabete 'O' strain were more pleomorphic than those of measles virus, 120-300 nm in diameter and contained helical nucleocapsids 17.5 nm in width. The external surface of virions were covered by a patchy 9 nm fringe. Tajima and colleagues (1971b) noted that rinderpest virus nucleocapsids viewed in negatively-stained preparations were indistinguishable from those of CDV.

The buoyant densities of CDV, measles and rinderpest virions have been variously determined by equilibrium centrifugation in sucrose, potassium tartrate (K-T), or cesium chloride (CsCl) gradients. Phillips and Bussell (1973) reported that the virions of the Onderstepoort strain of CDV were heterogeneous with respect to buoyant density. In both CsCl and K-T gradients, infectivity peaks at 1.231 gm/cm^3 were detected. Armitage and associates (1975) centrifuged CDV virions to equilibrium in sucrose and K-T gradients. A peak of infectivity was detected at 1.195 gm/cm^3. In CsCl gradients, a peak was detected at 1.24 gm/cm^3. Fractions detected at a density greater than 1.24 gm/cm^3 were attributed to the presence of disrupted particles with higher proportions of nucleocapsid to envelope that in intact virions.

In this study, fractions in which membrane-associated aggregates of nucleocapsids were detected ranged in density from 1.24-1.27 gm/cm^3 in sucrose. These values generally agree with results cited above for other morbilliviruses. Based on these results, intact particles would be expected to band at a density of approximately 1.23 gm/cm^3 and pure nucleocapsids at approximately 1.30 gm/cm^3. The observed density of nucleocapsids associated with disrupted cellular membranes would be expected to be intermediate between the two values.

Part VI

Comprehensive Discussion

Results of the electron microscopic, cytologic and antigenic studies reported in parts II, III and V of this dissertation support the classification of 72-P-535 within the morbillivirus genus of the family paramyxoviridae. As such, 72-P-535 represents the first morbillivirus isolated from North American cattle. Additional information reported in part IV suggests that California MCF isolates 76-R-911, 76-R-913 and others are members of the bovine syncytial virus group. They therefore represent the first viruses of this group reported from cattle in the western United States. The 77-R-941 isolate represents a multiple mixed infection involving a bovine syncytial virus and a virus related antigenically to 72-P-535. Additional investigations of this isolate are required to confirm these findings and further characterize the viruses involved.

Factors contributing to the strict association of infectivity with 72-P-535-infected cells were not investigated although certain pertinent observations were made. Extracellular particles appeared morphologically abnormal in several respects which may relate to the release of non-infectious virus. The apparently aberrant assembly of extracellular particles was frequently observed. Some particles contained multiple nucleocapsids while others appeared empty or contained cellular components. Additionally, the external fringe of released particles was frequently patchy or absent. To further investigate the factors contributing to the lack of infectivity of released particles, experiments addressing

the possible role of temperature sensitivity, formation of defective interfering particles, thermal lability of released virus and host-cell derived modifications of virus are required. In conjunction with these experiments, studies on the types and sizes of virus-specific RNA and protein species would prove helpful.

The results of these investigations do not prove that 72-P-535 causes MCF although the reported biological and pathological characteristics of morbilliviruses require that this possibility be investigated further. Morbilliviruses are lymphotropic and often affect the proper functioning of the cell-mediated immune system. They may also cause chronic rather than acute infections due, in part, to the ease with which they establish persistent infections in man and other animals. The combination of these characteristics suggests that a persistent infection of lymphoid cells could, by a variety of immunologically-mediated mechanisms, produce a lymphoproliferative disease with the pathological characteristics of MCF. Long term studies of animals inoculated by a variety of parenteral routes and the subsequent development of a laboratory animal model are crucial to the further investigation of this possibility.

Since rinderpest virus is the only bovine pathogen within the morbillivirus genus, the possibility that 72-P-535 may be a rinderpest virus variant must be addressed. Historically, large numbers of cattle were imported into the United States from enzootic areas before the enactment of current

importation and quarantine restrictions. It is somewhat surprising, therefore, that rinderpest has never been reported in the United States. Since an inapparently infected animal would not show clinical signs of disease, it is possible that a rinderpest virus variant which causes clinically silent infections could have escaped detection and be present in certain cattle populations. Such a hypothesis is supported by reports that in enzootic areas rinderpest virus strains vary widely in their virulence for cattle. Inapparently infected cattle have been reported in which the virus persisted in the same tissues from which 72-P-535 was isolated. The circumstantial implication of sheep as reservoirs of the agent responsible for MCF should be considered with respect to the ability of rinderpest virus to alter its host range and establish clinically silent infections in sheep and small ruminants. Thus, the host range, pathogenic properties and tissue tropisms of rinderpest virus suggest that the original hypothesis requires further investigation by the direct comparison of the two viruses. Unfortunately, such a comparison is not currently possible in this laboratory.

The isolation of a cell-associated morbillivirus from cattle may also be significant with respect to the recent virologic and epidemiologic findings which suggest that certain chronic degenerative diseases of man, notably SSPE and multiple sclerosis, may be due to a zoonotically transmitted morbillivirus. In Germany, such a possibility was suggested by Bachmann and colleagues after their isolation of a

morbilli-like virus (V-107) from cattle. Biological and antigenic comparisons of V-107, 72-P-535 and human SSPE viruses are currently underway.

Much has been learned concerning the morphogenic, cytopathogenic and antigenic characteristics of 72-P-535 and other viruses recovered from MCF cattle. Many questions remain and other isolates await analysis. The etiology and pathogenesis of MCF are complex interrelated problems which require innovative multidisciplinary approaches in their investigation. It is hoped that the information reported in this study will contribute to the eventual resolution of these questions.

Part VII

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